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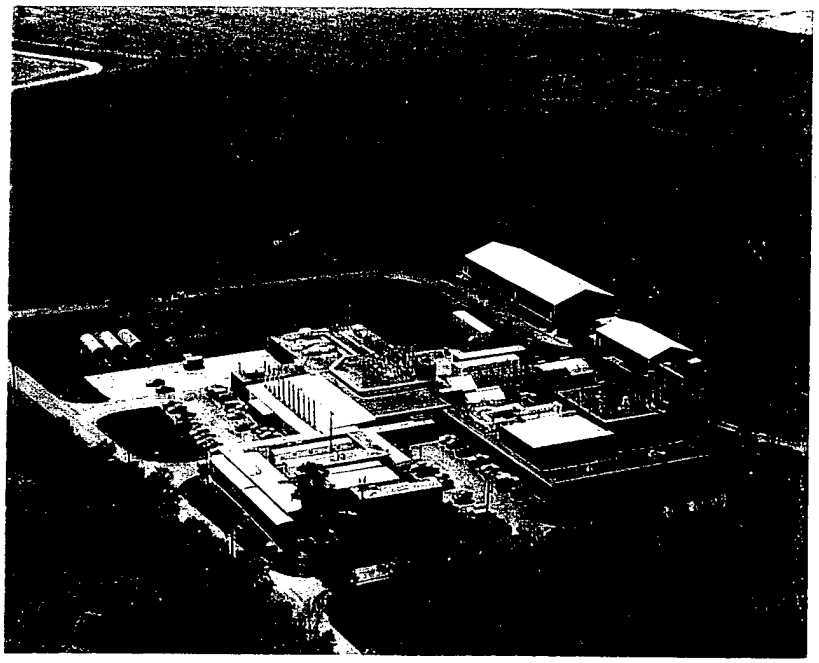

Task 92-29: Efficacy Assessment of Topical Skin Protectants Against Sulfur Mustard Vapors in Hairless Guinea Pigs

To

U.S. Army Medical Research and Development Command

September, 1997

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Olson, Carl T., D.V.M., Ph.D.; Snider, T. H.; Nitz, H. W.; Johnson, J. B.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Battelle Memorial Institute
Columbus, Ohio 43201-2693

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The objective of this work was to determine the dose-response relationship between the duration of a topical exposure of sulfur mustard vapors to hairless guinea pigs (HGPs) and the incidence of microblisters observed histologically. The dose-response curve was compared to results of similar work performed at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD). The effects of ketamine hydrochloride anesthesia on HGP skin reflectance were also examined.

The exposure time needed to produce a 50 percent incidence of microblisters in HGP skin exposed to saturated HD vapors was approximately 7.5 min. Comparison with USAMRICD study results indicated that the HGPs used in the present study were more tolerant of HD vapors by approximately 2 min. However, the probit analysis slopes exhibited the same rate of increase in microblisters as a function of HD vapor exposure time. The model was thus validated for use in screening prophylactic and therapeutic treatments against mustard injury. Ketamine hydrochloride anesthesia significantly reduced the baseline (pre-dose) level of red chromaticity on HGP skin test sites.

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PRINCIPAL INVESTIGATOR: Carl T. Olson, D.V.M., Ph.D.
T. H. Snider, H. W. Nitz, J. B. Johnson

CONTRACTING ORGANIZATION: Battelle Memorial Institute
Columbus, Ohio 43201-2693

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A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program

on

TASK 92-29:

Efficacy Assessment of Topical Skin Protectants Against Sulfur Mustard Vapors in Hairless Guinea Pigs

to

U.S. ARMY MEDICAL RESEARCH
AND MATERIEL COMMAND

September, 1997

Mr. T. H. Snider
Mr. H. W. Nitz

Battelle
Medical Research and Evaluation Facility (West Jefferson)
505 King Avenue, JM-3
Columbus, Ohio 43201-2693

In conducting the research described in this report the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health (NIH), Publication No. 86-23, revised 1985).

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FINAL REPORT

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TASK 92-29

Efficacy Assessment of Topical Skin Protectants Against Sulfur Mustard Vapors in Hairless Guinea Pigs

to

U.S. Army Medical Research
and Materiel Command

September, 1997

Thomas H.Snider,
Study Director

John B. Johnson, D.V.M.
MREF Manager

Executive Summary

The objective of Medical Research and Evaluation Facility (MREF) Task 92-29 was to establish at Battelle the hairless guinea pig (HGP) model for evaluating the dermatotoxic effects of sulfur mustard (HD) vapors and to screen the efficacy of several topical skin protectants. The task underwent modification in scope and changes in technical methods that necessitated retiring the original protocol and writing a revised version. The new objective was to validate the HGP model at the MREF by performing a HD dose-response study and comparing the incidence of microblisters with that observed in a study performed at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD). A final modification called for using the model to assess the efficacies of several systemic prophylactic and therapeutic materials against an HD vapor challenge.

This project suffered a 2-year interruption due to an outbreak of listeria in the only domestic colony of HGPs. Once the supply of HGPs was re-established, the HD dose-response study was completed, along with a determination of the effects of anesthesia on HGP skin reflectance. Instructions to terminate this task preceded its use as a screening paradigm.

Ketamine hydrochloride anesthesia significantly reduced the baseline (pre-dose) level of red chromaticity on HGP skin test sites. The effective HD dose, in terms of exposure time, to a saturated vapor required to produce a 50 percent incidence of microblisters, was approximately 7.5 min. Comparison with USAMRICD study results indicated that the HGPs used at Battelle were more tolerant of HD vapors. However, the probit analysis slopes exhibited the same rate of increase of microblisters as a function of HD vapor exposure time. The model was validated at Battelle, and could be used for screening prophylactic and therapeutic treatments.

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TASK 92-29:

Efficacy Assessment of Topical Skin Protectants Against Sulfur Mustard Vapors in Hairless Guinea Pigs

1.0 Introduction

Personnel of the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) have considered using the hairless guinea pig (HGP) for studying the dermatotoxicity of sulfur mustard (HD) vapors[1]. The HGP model has been used for evaluating candidate topical skin protectants (TSPs) for prophylaxis against HD. Double-sided tape pads with holes (one per pad) are affixed to treatment sites marked on anesthetized HGPs. Prescribed volumes of a TSP are applied to the skin within the tape holes. A paper-lined plastic cap is wetted with HD, inverted, and placed over each tape pad, thus producing a small chamber of HD vapors while preventing direct contact of liquid HD with the skin. After a prescribed exposure period, the vapor cap assemblies (tape pad and vapor cap) are removed and decontaminated, and the treatment site cleaned. Erythema is assessed before and after exposure to HD vapors with a skin reflectance meter[2], and tissue damage is assessed by histopathology. Scientists at USAMRICD have shown that the HGP model exhibits microvesication that is directly related to HD exposure time[3] and, that the HGP could be used to screen both TSPs[4] and systemic prophylactic and therapeutic (P&T) treatments[5, 6, 7] for HD vapors.

The objectives of Task 92-29 were to establish the HGP model at Battelle's Medical Research and Evaluation Facility (MREF) and to further develop the HGP model in order to evaluate TSPs against topical exposures to HD vapors. This was to be accomplished over the course of three phases outlined in the original proposal issued on February 11, 1993:

- Phase I (Anesthetic Effect Pilot Study) - Determine the significance of HGP skin blanching associated with general anesthesia as reported by USAMRICD, and determine whether HD remains on the HGP after TSP removal. Information from this phase was to be used to help decide whether exposed HGPs would have to be manually restrained during

reflectance readings or could be anesthetized, and whether the readings could be performed at the face of the hood. If the anesthetic effect is not significant, or if there is evidence of HD offgassing, then exposed HGPs would be anesthetized prior to making reflectance readings, thus reducing the possibility of escape from the fume hood. If the anesthetic effect on skin reflectance is significant, HGPs would have to be manually restrained during readings to avoid the blanching effect.

- Phase II (Validation: Dose Response Study) - Validate the model at the MREF by performing an HD vapor dose-response study with HGPs in each of two weight ranges and compare the results with data from similar studies conducted at USAMRICD. The objective of this phase was to determine HD vapor exposure periods that produced an incidence of microvesication ranging from 0 to 100 percent.
- Phase III (TSP Tests) - Determine an optimal HD vapor exposure period for the evaluation of up to 12 TSPs.

A memorandum from USAMRICD dated February 25, 1993 recommended cancellation of Phase I (Anesthesia Effect Pilot Study) and outlined modified methods for use in the other phases. Authorization to begin work on this task was received on May 18, 1993. MREF Protocol 84, "Dose Response Curves in Hairless Guinea Pigs Exposed to Sulfur Mustard Vapors," was signed on May 21, 1993. A USAMRICD memorandum dated July 1, 1993 eliminated the HD dose-response study using large guinea pigs (600 to 700 g). A team of Battelle investigators visited Dr. E.H. Braue at USAMRICD in July 1993 to observe the most recent techniques being used for TSP screening. Developments in the methods and endpoints used to assess TSPs with this model necessitated retirement of Protocol 84 and replacement with Protocol 99, entitled "Efficacy Assessment of Topical Skin Protectants Against Sulfur Mustard Vapors in Hairless Guinea Pigs" (Appendix A), which was signed in September 1993.

On September 29, 1993, Charles River Laboratories issued a bulletin stating that their HGP colony was exhibiting signs of listeriosis, and that they would not be able to supply any HGPs until their colony was re-established. Since the Charles River facility (Lakeview, NJ) was the sole source of HGPs within the United States , MREF personnel inquired whether foreign sources, Biological Research Laboratories Ltd (BRL; Basel, Switzerland) or High Oak Ranch Ltd

(Ontario, Canada), could meet research needs. Both facilities were in colony-building phases and orders were backlogged. BRL would not be able to supply HGPs until February 1995. Work on this task was suspended pending either the availability of HGPs or the development of an alternative animal model for screening TSPs.

HGPs, in limited numbers, became available from Charles River Laboratories in June 1995. In consultation with the USAMRICD Contracting Officer's Representative (COR), an assessment of the dermal sensitivity of these HGPs to HD vapors was deemed necessary prior to screening TSPs. Phase II (Validation: Dose Response Study) commenced on June 14, 1995. During a visit on June 27 and 28, representatives from USAMRICD requested that Battelle complete Phase II and then submit a new proposal for a change in scope of work from screening TSPs to screening up to 10 systemic P&T compounds for HD. Phase II exposure was completed on August 17, 1995. A revised proposal for developing the model for screening P&T treatments was submitted on November 1, 1995.

In 1996, Battelle received notice from USAMRICD that work on several tasks, including Task 92-29, should be halted and final reports written. This report describes the procedures used and the results obtained from the dose-response study performed between June and August, 1995.

2.0 Materials and Methods

Materials and methods employed in this study are described in MREF Protocol 99, Appendix A to this report. A brief overview is supplied in the following.

2.1 Chemical Surety Materiel

HD was supplied by the USAMRICD. The mean purity of undiluted HD used in this task was approximately 91.5 percent. Samples of HD were analyzed by MREF chemists prior to use on study. Also, at the completion of each dosing session, a 10-μL sample of HD was dispensed, from the device used to dose HGPs, into a 10-mL volumetric flask, and the flask was filled to the quantity sufficient line with hexane. After the volumetric flask was capped and mixed by

inversion several times, samples were aliquoted into glass vials for analysis of concentration by gas chromatography. The analyses, expressed as a percent of the expected concentrations, are presented in a dose control chart with 95 percent upper and lower confidence limits in Figure 1 (Appendix B). The mean of all dose control samples was 94 percent of the expected concentrations. Concentrations of all dose control samples were within the 95 percent confidence limits.

2.2 Vapor Cap Assemblies

Vapor cap test assemblies were fabricated before the day of dosing. A 30 x 2.5-cm strip of release paper was taped at its corners to the top of a clean laboratory work bench. A 30 x 2.5-cm strip of double-sided adhesive, laminated fabric carpet tape with release paper top, was placed on the release paper taped to the work bench. This formed a three-layer assembly with carpet tape protected on both sides by release paper. A 30 x 1-cm strip of tractor alignment edging from computer paper was affixed to each side of one edge of the tape strip so as to form a nonadhesive pull tab. The tape assembly was cut into sixteen 2.5 x 1.8-cm pads, each of which were subsequently perforated with a 12-mm diameter hole. These tape pads were stored in a sealed plastic bag until used. A 14-mm diameter disk of Whatman paper No. 2 was pressed into the inside top of a plastic cap (Columbia Diagnostics, Inc., Springfield, VA) with approximate dimensions of 17 mm OD, 14 mm ID, and 6 mm in height. These vapor caps were stored in a sealed plastic bag until used.

2.3 Test Animals

CRL:IAF/HA(hr/hr)BR male HGPs, 300 to 400 g, were obtained from Charles River Laboratories. Purina guinea pig chow and tap water were provided *ad libitum* to HGPs housed in groups of three in polycarbonate cages equipped with automatic watering systems. On the day before use, the back of each HGP was wiped with a gauze pad soaked in a 1:20 dilution of mild dish washing detergent in distilled water and then dried with another gauze pad. On the day of

dosing, each back was cleaned with distilled water and dried. Eight approximately 20-mm square test sites were demarcated along the dorsal midline in a 2 x 4 pattern and labeled A through H using an indelible-ink pen.

The HGPs were manually restrained, and estimates of baseline skin reflectance were recorded for dose sites A, B, E, F, G, and H with a chromameter (Minolta model CR-200, Ramsey, NJ). Each dose site was evaluated with a single flash of light from the chromameter. Sites C and D were omitted because they were anatomically similar to sites A and B. Since safety concerns prohibited removal of the chromameter probe from the hood without proof of decontamination, the baseline reflectance readings were performed at the face of the hood. The reflected light was converted into a three-coordinate system (L^*, a^*, b^*), in which L^* represented levels of brightness between white (+100) and black (-100), a^* represented the degrees of red (+60) versus green (-60), and b^* represented the degrees of yellow (+60) versus blue (-60). Only the red index parameter values were used in this study. Each HGP was anesthetized with a xylazine/ketamine mixture administered by intramuscular injection, and then restrained on a tie-down board. At approximately 10 min after the anesthetic injection was given, skin reflectance estimates at the same six sites were recorded again. Skin reflectance readings were not taken following dosing with HD vapor because this would have involved the use of the chromameter inside the fume hood. A MREF requirement that all equipment placed in a fume hood undergo proof of decontamination prior to use outside the hood prevented collecting post-dose readings.

The release paper was pulled off one side of a tape pad, and the tape pad pressed onto one of the eight test sites demarcated on a HGP. The other sites were similarly prepared, and the HGP was transported into a fume hood for dosing.

2.4 Administration of HD

On each of eight test days, seven HGPs were dosed into two sets of three and one set of one. The top release paper was removed from each test site tape pad in a set of HGPs. Filter paper in the top of each of eight vapor caps per HGP was dosed with approximately 10 µL of HD and the cap inverted on a glass slide to allow partial vaporization of the dose within the cap. Just before a cap was to be applied onto a test site, it was placed onto a carrying device to minimize

loss of HD vapors. Each cap was transported near a HGP, and at approximately 7 min after HD was dosed, removed from the carrying device, positioned onto the center of a test site, and pressed lightly to make a seal between the cap and the tape pad.

A dosing schedule that dictated when each vapor cap was dosed, placed on a test site, and removed was followed. On every HGP, the process control site (H) received a 4-min exposure. HD vapor exposure durations were rotated among the other seven sites from animal to animal to remove any confounding effect of position with dose. Results from the H site of HGPs were used to determine whether individual sensitivity of HGPs to HD vapor varied significantly and to establish a database for future process quality control. During the first four test days, the nominal exposures at sites A through G ranged from approximately 3 to 9 min. Histopathologic results indicated less than 100 percent incidence of microvesication in each of the exposure groups for the first four days of testing. The exposure range was increased to 7 to 13 min for the last four test days. After the prescribed exposure period, each tape pad with vapor cap attached was removed with forceps and submerged into decontamination solution.

2.5 Tissue Sample Collection and Processing

Each animal was anesthetized with halothane at approximately 24 hr following exposure, and to perform euthanasia, the thoracic cavity was surgically opened to produce a pneumothorax. Dermal specimens were collected from the center of each test site, and from an untreated control site posterior to sites G and H at the dorsal midline. Each specimen retained identity by placement into a labeled cassette. All samples were held at the MREF for at least 24 hr before histologic processing. After fixation, they were processed by routine paraffin embedding, sectioning at approximately 5 μm, and hematoxylin and eosin staining, and examined by light microscopy. Each specimen was evaluated for epithelial necrosis, follicular necrosis, microblisters, pustular epidermitis, dermal necrosis, vascular necrosis, and hemorrhage. Each histopathologic parameter was scored both quantally (1 for the presence of each endpoint and a 0 for its absence) and semi-quantitatively for endpoint severity (0 to 4 for none, mild, moderate, marked, and severe). The slides were then submitted to Dr. E. H. Braue at USAMRICD for confirmation of histopathologic evaluations.

2.6 Statistical Analyses

Skin reflection red index estimates at each dose site taken after anesthesia were subtracted from estimates taken before anesthesia, resulting in skin reflection differences (SRDs). Each SRD was normalized by the mean of the baseline readings at that site and the site contralateral to it to form a parameter representing skin reflectance relative change (SRRC). Skin reflectance data were recorded in a notebook spreadsheet program (Quattro Pro 6.0, Novell, Inc), as were histopathologic data. Descriptive statistics for SRRCs and percent incidence and severity of histopathologic endpoints were tabulated using the Statistical Analysis System (SAS Institute, Cary, NC).

A probit dose-response model in logarithm base 10 of HD exposure time was fitted to the percent incidence data for microblisters. Dose-response percentiles were estimated from this model. The ED_{50} was defined as the HD vapor exposure time that would produce microblisters on 50 percent of observed skin sites. For comparison, a probit dose-response model in logarithm base 10 of HD vapor exposure time was fitted to the percent incidence of microblister data from a USAMRICD study published in 1992 by Dr. E. H. Braue *et al.* All tests were conducted at the 5 percent significance level.

3.0 Results

Tables are presented in Appendix C. On the first test day, three HGPs were inadvertently treated for approximately 1 min longer than was intended. Although this somewhat sullied the symmetry in what was a completely randomized block design, it did not detract from the validity of the probit analysis since the entire range of HD vapor exposure periods ranged from 3 to 13 min. Also on the first test day, baseline skin reflectance readings were collected but post-anesthesia readings were inadvertently not taken. On the seventh test day, one HGP with a prolapsed rectum was not dosed. Thus, the total number of HGPs used in this study was 55.

3.1 Skin Reflectance

Tables of individual HGP red index skin reflectance results are presented in Tables 1 through 8 for the eight test days. Table 1 has only baseline readings for the first test day, but Tables 2 through 8 include both baseline and after-anesthesia readings, as well as calculations of SRRCs. The following schematic presents the mean and standard deviation of SRRCs for each test site measured across 49 HGPs examined both before and after dosing.

Skin Reflectance Relative Change in Red Mean (Standard Deviation)			
Site	Anterior		Site
B	0.41 (0.10)	0.41 (0.10)	A
D	*	*	C
F	0.46 (0.10)	0.41 (0.11)	E
H	0.43 (0.13)	0.45 (0.10)	G
Posterior			

* skin reflectance was not measured at sites C and D.

The mean SRRC of 285 sites evaluated was 0.43, representing a 43 percent decrease in the red color dimension relative to each HGP's baseline reading. The SRRC value ranged from 0.03 to 0.68 with a standard deviation of 0.11. The administration of ketamine anesthesia had a statistically significant ($p < 0.05$) blanching effect on HGP skin.

3.2 Histopathology

Table 9 presents the incidence of test sites that were scored positive for histopathologic endpoints as a function of HD vapor exposure period. The same data are presented graphically in Figure 2 for four endpoints that changed with exposure period, i.e., epidermal and follicular

necrosis, microblisters, and pustular epidermitis. Table 10 presents the average severity scores for each histopathologic endpoint by HD vapor exposure period. The same data are presented graphically in Figure 3 for the same four endpoints. Low background incidences of epidermal (approximately 7 percent) and follicular (approximately 2 percent) necrosis were observed in untreated control tissues. No other endpoint was observed in the untreated tissues. Epidermal and follicular necrosis were observed in nearly all treated tissues, but vascular necrosis was not evident. Epidermal necrosis ranged from approximately 92 percent with a mean severity of approximately 0.9 in the 3-min exposure group to 100 percent with a mean severity of approximately 3.6 in the 12-min exposure group. Follicular necrosis ranged from approximately 85 percent with a mean severity of approximately 0.9 in the 4-min exposure group to 100 percent with a mean severity of approximately 2.2 in the 12-min exposure group.

Microblisters were not seen in the 3- and 4-min exposure groups and increased in a dose-response fashion from approximately 6 percent with a mean severity of approximately 0.1 in the 5-min exposure group to approximately 96 percent with a mean severity of approximately 2.6 in the 12-min exposure group. Figures 4 and 5 present probit curves for microblister data collected at the MREF and at USAMRICD, respectively. Table 11 summarizes the results of the probit analyses for the two studies. The doses predicted to produce a microblister incidence of 50 percent (ED_{50}, with 95 percent confidence limits) were approximately 7.5 (7.2, 7.9) min for the MREF study and 5.3 (5.0, 5.5) min for the USAMRICD study. This represented a significant shift in the sensitivity of the HGPs to HD vapors. The slopes of the probit curves were approximately 8.9 (7.4, 10.5) for the MREF study and 10.9 (8.9, 12.9) for the USAMRICD study. These results suggest that the increasing rates of microblister formation in response to increasing HD vapor exposures were identical for the two studies, but shifted toward a lower sensitivity in the MREF HGPs. The MREF HGPs required an approximately 2-min longer exposure to exhibit the same effect seen in the USAMRICD animals. Additional dose-response studies would be needed to confirm whether the population of HGPs from Charles River Laboratories has truly become more tolerant to HD vapors, as this comparison suggests. Heavier HGPs have been reported to exhibit a higher degree of tolerance to HD vapors (personal communication with Dr. E. H. Braue). The ranges of body weights among the HGPs used in the

.two studies were 300 to 375 g (MREF) and 260 to 560 g (USAMRICD). The distribution of HGP body weights within these respective ranges may have had some bearing on the results.

Pustular epidermitis ranged from approximately 3 percent with a mean severity of approximately 0.0 (due to rounding) in the 4-min exposure group to approximately 26 percent with a mean severity of approximately 0.4 in the 11-min exposure group. Dermal necrosis was not evident until approximately 8 min of HD vapor exposure, at which time it was observed in approximately 7 percent of the samples with a mean severity of approximately 0.1, and increased to approximately 15 percent but with a mean severity of only approximately 0.2 in the 13-min exposure group. Hemorrhage was not evident until approximately 7 min of exposure, at which time it was observed in approximately 2 percent of the specimens with a mean severity of approximately 0.0 (due to rounding), and increased to an approximately 33 percent incidence but with a mean severity of only approximately 0.3 in the 12-min exposure group. Thus, although HD apparently penetrated into dermal layers with increasing frequency at longer exposure periods, its toxicity there was insignificant.

4.0 Conclusions

Ketamine anesthesia administered intramuscularly induced a profound blanching effect on HGP skin, decreasing the red index chromaticity endpoint by an average of 43 percent from pre-anesthetic baseline levels. This result indicates that subsequent to dosing, skin reflectance readings should be collected from HGPs that have fully recovered from the effects of anesthesia. Studies performed at USAMRICD have indicated that by approximately 30 min after removal of HD vapor caps, no detectable concentration of residual HD from HGP skin can be detected by gas chromatography. Thus, pending confirming evidence by MINICAMS analysis at the MREF, it may be possible to remove HGPs from the fume hood for skin reflectance measurements.

A series of HD vapor exposures ranging from 3 to 13 min produced a microblister dose-response curve that estimated a HD vapor exposure ED_{50} for microblisters of approximately 7.5 min, which was approximately 2 min longer than that determined in a similar study performed at USAMRICD. The probit slopes of data from the two studies were statistically equivalent,

suggesting the same processes for microblister formation as a function of HD vapor exposure time. Epidermal and follicular necrosis and pustular epidermitis also increased in frequency and severity with exposure period, but the results were not conducive to probit analysis. Dermal and vascular necrosis and hemorrhage were not relevant endpoints for assessing HD vapor damage. The best endpoint for assessing HD dermatotoxicity and its amelioration by prophylaxis and therapy treatments in the HGP appears to be the incidence of microblisters.

The HGP/vapor cap model for studying HD dermatotoxicity has been successfully transitioned to the MREF. Studies to confirm the safety of removing HGPs from a fume hood at a specified time after HD vapor cap removal are needed before reflectance readings can be made on unanesthetized HGPs.

5.0 Archives

Records pertaining to the conduct of Task 92-29 are contained in Battelle Laboratory Record Book 348 and are archived at the MREF. All original data will be maintained at Battelle until forwarded to USAMRMC. Duplicate sets of histopathology slides were produced, and one complete set was given to Dr. E.H. Braue of USAMRICD.

6.0 Acknowledgments

The name, role in the study, and highest academic degree of each of the principal contributors in this study are:

John B. Johnson	MREF Manager	D.V.M., M.S.
Thomas H. Snider	Study Director	B.S.
Harold W. Nitz	Lead Technician	Laboratory Animal Technologist

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APPENDIX A

MREF Protocol 99

Efficacy Assessment of Topical Skin Protectants Against Sulfur Mustard Vapors in Hairless Guinea Pigs

Study Performed by Battelle Memorial Institute
505 King Avenue
Columbus, Ohio 43201-2693

1. Principal Investigator and Manager: David W. Hobson, Ph.D., D.A.B.T., Medical Research and Evaluation Facility (MREF)

2. Study Director: Thomas H. Snider, B.S., D.A.B.T.

3. Study Veterinarian: Allen G. Manus, D.V.M.

4. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)

5. Sponsor Monitor: LTC Don W. Korte, Jr., Ph.D., U.S. Army Medical Research Institute of Chemical Defense (USAMRICD)

6. Objectives: This work will establish the hairless guinea pig as a model for studying treatment of HD vapor-induced injury at the MREF. A pilot study will be performed to determine (1) whether administration of anesthesia affects dermal irritation end points, and (2) whether residual HD vapors offgas from exposed and decontaminated hairless guinea pigs. This protocol will be used to assess the efficacies of both systemic prophylactic and therapeutic (SP&T) treatments and topical skin protectants (TSPs) against dermal exposures to HD vapors on hairless guinea pigs. A secondary objective is to determine dose response curves for sulfur mustard (HD) vapor in hairless guinea pigs.

7. Experimental Design: A method is presented for topical exposure of hairless guinea pigs to HD vapors, and for quantifying the dermal irritation response. Multiple test sites are used on each hairless guinea pig, including quality control sites for normalizing inter-animal variability responses. HD is delivered onto a paper disk pressed into the top of a plastic cap, which is adhered to the test site with double-faced tape. Degrees of erythema produced by varying responses to HD are estimated by the difference in skin reflectance (a* chromaticity parameter) before dosing (or before TSP application, if included) versus after dosing (and after TSP removal) at specified times. Reflectance measurements are adjusted by subtracting naive, pre-dose measurements for each skin site. In TSP efficacy tests, net reflectance changes are normalized to no-TSP control site

values. Procedures for estimating skin reflectance with the chroma meter are given in Battelle SOP MREF VI-004, "Use of the Minolta Chroma Meter CR-200 as an Indicator of Dermal Irritation Severity Following Topical Exposure to Vesicating Agents at the MREF." Each lesion is also measured in length and width to estimate the area of erythema.

This protocol includes methods for three types of experiments: (1) a dual-purpose pilot study to evaluate the effect of anesthesia on reflectance measurements and to estimate the degree of HD offgassing from the dorsal surface of exposed hairless guinea pigs, (2) efficacy tests, and (3) dose response studies. Treatment groups nominally consist of nine hairless guinea pigs on each of three replicate test days.

In the pilot study, eight sites per animal are exposed to HD vapors. Half of the dose sites are pretreated with the standard TSP. Skin is exposed to HD vapors for a period needed to produce moderate irritation. Skin reflectance is estimated before and after anesthesia administration, before dosing, and 4 hr after dosing. After decontamination, the animals are monitored for HD offgassing with a MINICAMs® according to procedures in MREF SOP III-022, "Standard Operating Procedure for Sampling and Analysis of Chemical Surety Materiel Using a MINICAMs® (CMS Research Corporation) Analyzer".

In TSP efficacy tests, an initial reflectance reading is made before application of the standard and test TSPs at two dose sites each. Two additional dose sites are used as naive controls. A single HD vapor exposure period is held constant across all treatment groups, and a final reading is made after TSP removal. In SP&T treatment efficacy tests, reflectance readings are made at prescribed times before and after exposure to HD vapors.

In the dose response study, eight sites per animal are exposed to HD vapors for the same time period. Exposures are varied across hairless guinea pigs within a range of time periods needed to produce a mean microvesication rate ranging from zero to 100 percent incidence. A dose response curve is determined for hairless guinea pigs within a prescribed weight range.

A. Test Systems - Hairless guinea pigs were specified for use in this study by the sponsor, who has previously demonstrated that they are suitable for multiple

challenges with neat chemical surety materiel (CSM) and other irritants.[1,2,3] The hairless guinea pig provides an area sufficient for percutaneous exposures at multiple test sites without the usual complications associated with a heavy hair coat typical of most breeds of rodents and lagomorphs.

(1) Animals - CRL:IAF/HA(hr/hr)BR male hairless guinea pigs, 8 per treatment group; Supplier: Charles River Laboratories

(2) Weight Range - 300 to 400 grams

(3) Quarantine - Hairless guinea pigs are held in isolation and observed for clinical illness for at least 7 days prior to study initiation. Quarantine may be performed at Battelle's King Avenue animal facility or at the MREF.

(4) Acclimation - All hairless guinea pigs are held at the MREF at least 24 hr prior to study initiation.

(5) Selection - Hairless guinea pigs that are in good physical condition after a minimum 7-day quarantine period are selected. Individuals are then selected for study on the basis of health and proper weight (between 300 and 400 g). Selected animals are randomly assigned to weight-homogenized treatment groups for use on study.

(6) Animal Identification - Ear tag or tattoo; positive identification is required for each hairless guinea pig upon admission to quarantine. Cage cards, at a minimum, give animal number, sex, supplier and date of receipt for each hairless guinea pig.

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[1]Mershon, M.M. et al. 1990. Hairless guinea pig bioassay for vesicant vapor exposures, Fund. Appl. Toxicol. 15, 622.

[2]Braue, E.H., Jr., Koplovitz, I., Mitcheltree, L.W., Clayson, E.T., Litchfield, M.R., and Bangledorf, C.R. 1991. Characterization of the sulfur mustard vapor induced cutaneous lesions on hairless guinea pigs. Submitted to Fund. Appl. Toxicol.

[3]Braue, E.H., Mershon, M.M., Wade, J.V., and Litchfield, M.R. 1990. In vivo assessment of vesicant skin injury using a Minolta Chroma Meter. J. Soc. Cosmet. Chem. 41:259-265.

(7) Housing - Before being used in experiments, hairless guinea pigs are housed in groups of three in polycarbonate cages equipped with automatic watering systems.

(8) Lighting - Fluorescent lighting, light/dark cycle is 12 hr each per day.

(9) Temperature - Maintained at 18-26 degrees. At least 90 percent of the total recordings will fall within the specified range.

(10) Humidity - Maintained at 40-70 percent. At least 90 percent of the total readings will fall within the specified range.

(11) Diet - Purina Certified Guinea Pig Chow pellets are available at all times. No contaminants are known to be present in the feed which would interfere or affect the results of the study.

(12) Water Supply - Water is supplied from the public water system and given ad libitum. No contaminants are known to be present in the water which would affect the results of the study.

(13) Laboratory Animal Welfare Practices - Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a research facility (Number 31-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurance regarding the Department of Health and Human Services policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health (NIH), on August 27, 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication Number 85-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animals Welfare Act of August 24, 1966 as amended.

(14) Accreditation - On January 31, 1978, Battelle Memorial Institute received full accreditation of its animal-care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since

the original accreditation. The MREF is a part of the facilities granted full accreditation.

(15) Animal Care During Test - All hairless guinea pigs are held singly in the MREF hood system in polycarbonate cages positioned on pads heated to a minimum of 41 C with circulating water. The cages contain bedding and are supported by acrylic tables 15 cm from the floor of the hood to prevent formation of eddies in front of the cages. After anesthesia, restraint, and treatment, hairless guinea pigs are removed from the restraint boards and returned to the heated polycarbonate cages for up to 4 hr after dosing. A plastic-backed diaper is placed under each anesthetized hairless guinea pig to prevent aspiration of bedding. Drinking water is made available ad libitum. Upon completion of the study, all surviving hairless guinea pigs are anesthetized with halothane via inhalation. In order to prevent recovery, the thoracic cavity is surgically opened on the ventral side. Samples of the treatment area skin may be harvested, and the animals are disposed of by incineration after proof-of-decontamination (POD).

B. Experimental Overview

(1) Outline of Studies - A pilot study, efficacy tests, and an HD vapor dose response study will be performed with this test system. The pilot study requires three replicate dosing days. Each efficacy test requires one day of dosing nine hairless guinea pigs per TSP. The dose response study requires eight test days of a minimum of seven hairless guinea pigs each, depending on the number of exposure periods. The total number of hairless guinea pigs to be used is anticipated to be a minimum of 316, but will increase if a complete range of microvesication incidence is not achieved within the first round of dose response studies. This number of animals will provide for 20 efficacy tests, including a 1/10 rate for hairless guinea pigs disqualified due to high baseline skin reflectance readings.

NUMBER OF HAIRLESS GUINEA PIGS REQUIRED PER TYPE OF STUDY PER DAY

Pilot Study Test Day	Efficacy Tests Test Day	Dose Response Study Test Day
1 2 3	1 . . 20	1 2 3 4 5 6 7 8
8 8 8	9 each day	7 7 7 7 7 7 7 7

(2) Definition of Treatment Groups

(a) Pilot Study - HD vapor exposure is held constant at 4 min, the standard exposure for use in efficacy tests. The study is conducted over three replicate days of eight hairless guinea pigs for each day, for a total of 24 animals. All hairless guinea pigs are treated the same, but half of the dose sites are pretreated with the standard TSP, ICD No. 1511, or as specified by the sponsor.

(b) TSP and SP&T Treatment Efficacy Tests - Each hairless guinea pig is pretreated with one TSP (along with the standard) or parentally administered treatment.

(c) Dose Response Studies - Initially, the exposures range from an anticipated minimum irritation level to an anticipated marked or severe irritation level. The seven initial exposure times are 3, 4, 5, 6, 7, 8, and 9 min. After all eight days of testing are completed, the incidence of microvesication is analyzed for each treatment group. If the mean incidence range does not include zero and 100 percent, then a new set of treatment groups may be assigned with shorter and/or longer exposure periods.

(3) Number of Animals Used Per Test Day - Placement of hairless guinea pigs into treatment groups is based on an algorithm that optimizes homogeneity of body weights among groups. The pilot study uses eight hairless guinea pigs per day. Animals to be used over the series of test days, that comprise a dose response experiment, are assigned to one of the treatment groups based on their body weights. The dose response study involves, nominally, seven hairless guinea

pigs per day. TSP and SP&T treatment efficacy tests are performed using nine hairless guinea pigs per day.

(4) Replicates - The pilot study is repeated over three days. The sample size of treatment groups for efficacy tests is determined by power calculations to determine test sensitivity. The number of hairless guinea pigs per efficacy test group is nine with two test sites each, for a total sample size of 18. Dose response studies are replicated across eight days to obtain an overall sample size of eight hairless guinea pigs for each of the seven exposure periods. At the study director's discretion, fewer than eight replicates may be used if the data on hand exhibit within-group variances small enough to allow discrimination among groups. Also at the discretion of the study director, the allocation of animals into treatment groups may be augmented by a sensitivity analysis program that determines where data are needed to optimally determine a probit function.

C. Test Articles

(1) Topical Skin Protectants - TSPs are supplied by the sponsor. It is the responsibility of the sponsor to ensure that appropriate identification (batch number, lot number, physical state, etc.), expiration date (if available), safety and storage data are supplied for each candidate TSP received by the MREF.

(2) Irritant

(a) HD is supplied by USAMRICD. Purity, appropriate identification (batch number, lot number, state), and stability data are supplied by USAMRICD. Purity and stability are confirmed periodically by Battelle.

(b) Surety, security, and safety procedures for the use of CSM are thoroughly outlined in facility plans, in personnel requirements for qualifications to work with agents, and in agent storage and use standard operating procedures. Specific procedures have been included in this document to ensure the safety of the personnel conducting this experiment.

D. Construction of Vapor Cap Test Assemblies

(1) Fabrication of the Tape Pad - A 30 x 2.5-cm strip of release paper is taped at its corners to the top of a clean laboratory work bench. A 30 x 2.5-cm strip of

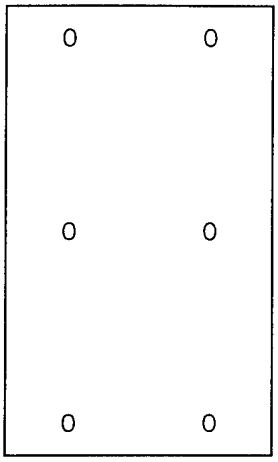
double-sided adhesive, laminated fabric carpet tape with its release paper on top, is placed over the bottom release paper. This forms a three-layer assembly of carpet tape protected by release paper. A 30 x 1-cm strip of tractor alignment edging from computer paper is affixed to each side of one edge of the tape strip so that a nonadhesive pull tab is formed. The assembly is cut into 16, 2.5 x 1.8-cm strips that subsequently are perforated with 12-mm diameter holes. These tape pads are stored in a sealed plastic bag until used.

(2) Fabrication of the Vapor Cap - A 14-mm diameter disk of Whatman paper No. 2 is pressed against the inside top of a plastic cap with approximate dimensions of 17 mm OD, 14 mm ID, and 6 mm height. Caps with these dimensions may be purchased from Columbia Diagnostics, Inc., 7942 Cluny Ct., Springfield, VA 22153 (Cat. No. P799C). These vapor caps are stored in a sealed plastic bag until use.

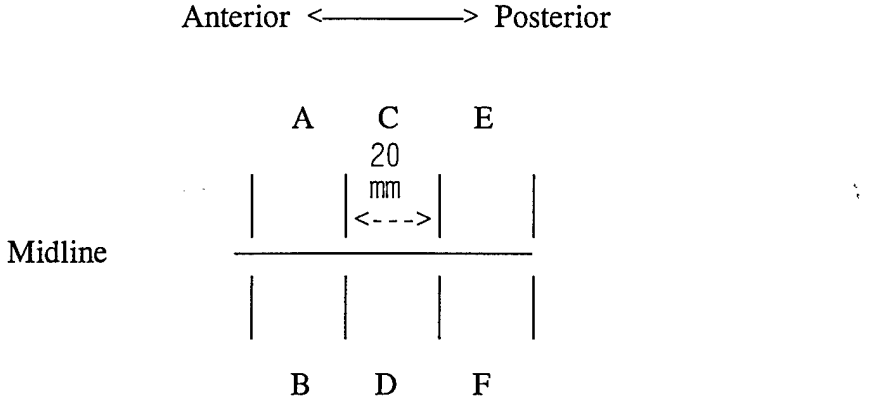
E. Preparation of Animals for Testing

(1) Cleaning the Dorsum - On the day before dosing, the back of each hairless guinea pig is carefully wiped with a 2 x 2 inch gauze pad soaked in a 1:20 dilution of a mild dish washing detergent in distilled water to remove soil and debris. A dry gauze pad is used to dry the dorsum. Approximately 1 hr before dosing, the dorsum is cleaned as before but with distilled water.

(2) Marking Test Sites - Each hairless guinea pig is removed from its holding cage and manually restrained while a marking template is centered on the midline at the anterior dorsum, and dose sites are marked. The template has six, 1-mm diameter holes at the corners and mid-edges of a 4 x 2-cm rectangle:



The tip of a marking pen is touched to the skin through these holes, and the resulting points define the anterior and posterior edges of two anterior dose sites on each side of a hairless guinea pig. Two more points are placed on the posterior dorsum to define the last two dose sites. The six test sites are designated A through F, as shown in the following schematic.



For the pilot study and dose response study, two additional sites, G and H, are marked posterior to E and F. The dose site dimensions may be altered if necessary to accommodate a larger chroma meter aperture than the one (11 mm diameter) typically used. In the pilot study and in TSP efficacy tests, TSPs are spread on pairs of test sites so that one side of the dorsum is a mirror image of the other side. The application sequence is rotated from animal to animal to remove any anterior-posterior positional bias from the results.

(a) Positive Controls - Two sites (either A and B, C and D, or E and F) each receive a standard, 4-min HD vapor exposure without any prior TSP application. The skin reflectance differences at the positive control sites are used to maintain quality control on an individual basis. The reflectance differences at all other test sites are divided by the mean of reflectance differences at the control sites to form reflectance difference ratios (RDRs) in order to normalize for individual sensitivities. The mean control reflectance difference is used to maintain quality control for individual sensitivity.

(b) Standard TSP - A standard TSP, identified by USAMRICD, is applied to two other, contralateral sites. Reflectance results at these sites are statistically compared with test TSP results to evaluate relative efficacy.

(c) Candidate TSP - The candidate TSP is applied at the remaining pair of contralateral sites.

(3) Systemic Prophylactic Treatments - These are administered either parenterally by injection or per os at a test-specified time, approximately 1 hr, prior to exposure to HD vapors.

(4) Baseline Skin Reflectance Readings - Hairless guinea pigs are held in butyl rubber-gloved hands in a dosing hood, and estimates of baseline skin reflectance are recorded for each dose site according to procedures in Battelle SOP MREF VI-004, "Standard Operating Procedure (SOP) for the Use of the Minolta Chroma Meter CR-200 as an Indicator of Dermal Irritation Severity Following Topical Exposure to Vesicating Agents at the Medical Research and Evaluation Facility (MREF)". Since the chroma meter probe cannot be removed from the hood without proof-of-decontamination, the baseline reflectance reading is performed in the hood. Any hairless guinea pig with a baseline a* chromaticity parameter reading that is greater than 13 is replaced. This ceiling was established by workers at USAMRICD and represents the threshold above which significant, treatment-related erythema is obscured. For prophylactic treatment efficacy tests, another reading is made at a test-specified time, nominally 1 hr after administration of the treatment.

(5) Anesthesia - Hairless guinea pigs are given 6.0 mg/kg (20 mg/mL) xylazine and 35.0 mg/kg (100 mg/mL) ketamine by intramuscular injection after baseline reflectance readings are recorded. For TSP efficacy tests, hairless guinea pigs

are anesthetized and treated in groups of three, with approximate 30-min intervals between groups. The time of anesthetic administration is recorded. Hairless guinea pigs are then placed in a prone position on holding boards and restrained by taping down each leg with 1/2-inch wide cloth tape. Ketamine anesthetic boosters of the same dose are administered as needed for the entire period of restraint on the boards.

(6) Body Temperature Maintenance - The hairless guinea pigs are warmed by a pad heated to a minimum of 41 C with circulating water for their entire duration in the hood. After anesthesia and restraint, the hairless guinea pigs are placed on a heated pad, and a cardboard hood followed by a plastic-backed diaper are placed over each hairless guinea pig to help retain body heat. The covering is removed during TSP application and while the HD vapor cap is affixed. The cardboard hood is discarded after decontamination, but a diaper is placed over each hairless guinea pig during recovery. In addition, the cage top is inverted, and a large, plastic-backed diaper is loosely taped over it to prevent heat loss.

F. Preparation of Test Sites for Vaporous HD Challenge

(1) Before applying a tape pad to a test site, the lower piece of release paper is pulled away from one side. The tape pads are placed with the long axis perpendicular to the hairless guinea pig's spine and with the pull tabs oriented laterally. Tape pads are applied to sites A through F (and to sites G and H for the pilot study and the dose response study). The perforation in each pad is centered over a dose site and lightly pressed into place.

(2) For the pilot study and TSP efficacy tests, a TSP is applied as required per test to the dose sites.

(a) Each TSP material, standard or candidate, is applied at a calculated uniform depth of approximately 0.2 mm (application rate = 0.02 mL/cm^2) to standardize application conditions. A 100-μL syringe (no needle) is used to deliver a target volume of 22.6 μL of the TSP inside each tape pad well. The application depth may vary at the direction of the sponsor.

(b) A spatula is used to uniformly spread each TSP material within the 1.2-cm diameter well to obtain a smooth and even coating. Care is taken to minimize any loss of material on the rim of the tape well. The time of application is recorded.

(c) Each TSP material is allowed to remain on the pretreatment area for 15 min before vapor cap application. Different wear times may be specified by the specific test requirements.

(d) The top layer of release paper is pulled away from the carpet tape just before vapor cap application.

G. Application of HD to Animals

(1) Exposures of HD vapors are made in fume hoods approved for use with chemical surety materiel. During dosing and throughout the exposure period for each test, hairless guinea pigs are positioned inside hoods to maintain air flow of approximately 100 linear ft/min, anterior to posterior, over the hairless guinea pig. Besides ensuring personnel safety, this positioning helps to eliminate the possibility of hairless guinea pig intoxication by inhalation.

(2) Applications of HD are made at test-specified times and consist of a constant volume of application. The challenge dose volume may be changed at the direction of the sponsor and/or study director. All safety procedures given in Battelle SOP MREF I-002, "Standard Operating Procedure (SOP) for the Storage, Dilution, and Transfer of GA, GB, GD, TGD, VX, HD, HD/L, and L When CSM Concentration/Quantity is Greater Than Exempt Levels", are observed during handling and dosing of HD. Instructions for applying HD into vapor caps are specified in Battelle SOP MREF II-003, "Percutaneous Application of Either Liquid or Vaporous HD, L, and HL Chemical Surety Materiel to the Dorsum of the Hairless Guinea Pig to Test Defensive Methods Material."

A vapor cap assembly is inverted and placed in a well drilled in an aluminum block. A 10-μL (or other, sponsor-specified) volume of HD is dispensed from a syringe at the center of the paper in the cap. A Hamilton 7001N syringe with a sharp-tip, positive displacement needle may be used to provide a point source, air-dropped delivery. A larger syringe may be used in a calibrated micrometer-driven dosing device (MDDD) to administer HD. Alternatively, a calibrated pipettor such as a 100-μL capacity Pipetman may be used with a disposable pipet tip. If a droplet of HD remains on the end of the needle or tip, the needle or tip may be brought down close to the paper surface so as to "wick" off the droplet.

(3) Immediately after application, the dosed vapor cap assembly is lifted with forceps out of the block, inverted, and placed on a glass microscope slide. Eighteen vapor caps are thus dosed, inverted, and placed on six glass slides. All upper release papers are removed from the current set of three hairless guinea pigs. At approximately 2- to 3-min after dosing, placement of the assemblies begins with approximately 10-sec intervals between dose sites. Each cap is lifted with forceps, centered over the hole in the tape pad at a dose site and placed on the tape pad. The cap top is lightly pressed with forceps to ensure a seal between the lower rim of the cap and the carpet tape.

(4) The exposure sites are dosed in alphabetical order with a 10-sec interval between each dose. Times of dosing are recorded.

(6) Exposure Periods

(a) Pilot Study - The exposure period to HD vapors for all eight test sites (A through H) is a minimum of 4 min. This exposure may be altered to determine the effect of anesthesia associated with other exposure periods. Full anesthesia is administered at approximately 4 hr after dosing, and reflectance estimations are made just before and approximately 10 min after administration of anesthesia.

(b) TSP and SP&T Treatment Efficacy Tests - The nominal period for HD vapor exposure is 4 min. This exposure time may be altered by the study director in order to produce a different vesicant challenge that improves discrimination among the TSPs or SP&Ts tested.

(c) Dose Response Studies - The exposure period for all eight test sites (A through H) to HD vapors varies per test specifications. This may extend for up to 4 hr after vapor cap application, but shorter times may be selected depending on the anticipated desired response. The initial exposure periods are approximately 3, 4, 5, 6, 7, 8, and 9 min. Exposure periods may be altered by the study director in order to produce a full range of microvesication incidence across treatment groups and adequately determine a dose response.

H. Study-Specific Decontamination - Battelle SOP MREF II-003, "Standard Operating Procedure (SOP) for the Percutaneous Application of Either Liquid or Vaporous HD, L, and HL Chemical Surety Materiel to the Dorsum of the Rabbit to Test Defensive

Methods Material", details procedures for removal and decontamination of vapor caps. A solution of 5 percent sodium hypochlorite (NaOCl) is used for all HD decontamination. At the specified time, each vapor cap is grasped with a pair of forceps and placed into a bucket of decontamination solution. After the last vapor cap is removed, forceps are used to lift the tape pad by the pull tab away from the skin and place it into decontamination solution.

(1) At the direction of the sponsor, test site skin is not decontaminated following exposure to HD vapors and removal of vapor caps. For systemic therapeutic treatment efficacy tests, the treatment is administered at a test-specified time, nominally 1 hr after the first vapor cap was applied on each animal.

(2) After vapor cap and tape pad removal, the animal is released from the restraint board and placed alone in a clear polycarbonate cage in the hood for the remainder of the study period. A plastic-backed diaper is placed under each anesthetized hairless guinea pig to prevent aspiration of bedding. Body temperature is maintained by placing a second diaper over the anesthetized hairless guinea pig, inverting the cage top, and draping a large diaper over it.

(3) For TSP efficacy tests, TSP is removed from each test site after a specified period, nominally 3 hr. Each hairless guinea pig is removed from its polycarbonate cage by a worker wearing a pair of butyl rubber gloves over a pair of latex gloves, and butyl apron. The weight of the animal is supported by the hood floor while the technician grasps the hairless guinea pig's head between a thumb and forefinger to calm and prevent the animal from lurching forward. A water-soaked Texwipe® swab is wiped over the test site and discarded into decontamination solution, followed by a dry Texwipe® swab. The hairless guinea pig is returned to its cage in the hood.

I. End point Measurement Procedures and General Decontamination

(1) Irritation Assessments - The end points used to quantify dermal irritation are the difference between pre-dose and post-dose skin reflectance readings, and other grossly observable indices of dermal irritation such as lesion area estimates. Reflectance readings are made on awake hairless guinea pigs at approximately 4 hr after dosing. Each hairless guinea pig is removed from its polycarbonate cage by a worker wearing a pair of butyl rubber gloves over a pair of latex gloves, and butyl apron. The weight of the animal is supported by the hood floor while the technician grasps the hairless guinea pig's head between a thumb

and forefinger to calm and prevent the animal from lurching forward. Reflectance readings are made on each of the test sites according to procedures in Battelle SOP MREF VI-004, "Standard Operating Procedure (SOP) for the Use of the Minolta Chroma Meter CR-200 as an Indicator of Dermal Irritation Severity Following Topical Exposure to Vesicating Agents at the Medica; Research and Evaluation Facility (MREF).

(2) Gross Lesion Evaluation - After skin reflectance determination is completed, each test site is evaluated grossly and rated for the presence of erythema, edema, and blister formation. Scorings are made according to the scheme shown below:

Appearance of Skin	Rating
Normal	O
First signs of erythema	E-
Definite erythema	E
Raised (edematous) erythema	E+
Frank blister	B
Pinpoint vesication	PV

(3) Dye Injection - This step may be included as part of the test to enhance lesion area estimations. After the 24-hr reflectance reading, each animal is given a 2.0-mL intramuscular injection, in each thigh, of a 3 percent suspension of trypan blue dye in saline. The dye requires at least 2 hr to translocate throughout the damaged vessels of the exposure areas. The dye forms a dark blue marking of the lesion against the contrasting pale blue of adjacent normal skin. A pink halo may extend 2-4 mm wider than the blue zone, which presumably is indicative of active hyperemia.

(4) Pathology - Histopathological evaluation of exposed skin is performed for the dose response study only. Each animal is anesthetized by halothane inhalation in a sealed glass container. To prevent recovery, the thoracic cavity is surgically opened on the ventral side. Dermal specimens are collected from the center of each test site. Each specimen is identified by placing it into a labeled jar or cassette. Specimens are identified by task number, charge account number, study director, date and time of tissue harvesting, and hairless guinea pig number. A warning label stating that the skin samples were exposed to HD vapors is affixed to the outside of each container and to the outside of the box

used for transportation. All samples are retained at the MREF for 24 hr before being transported to another facility for histologic processing. After fixation, they are processed for routine hematoxylin and eosin staining and histopathologic evaluation by light microscopy. Each specimen is evaluated for microvesication, intracellular edema, pustular epidermatitis, and follicular involvement (necrosis), and receives a score of 1 for the presence of each marker and a 0 for its absence. The histologic slides are sent with the report to the sponsor for confirmation.

(5) General Decontamination - All animals dosed with HD receive a general decontamination just prior to their removal from the hood system. After euthanasia, the remaining back of each animal carcass is decontaminated with a soaking wipe of 5 percent NaOCl. Carcasses are placed in double plastic bags which are sealed, and removed from the hood for POD and disposal by incineration.

J. Statistical Methods

(1) Baseline Subtraction - Skin reflection estimates taken after dosing are subtracted from estimates taken before the prescribed exposure at each dose site, resulting in skin reflection differences (SRDs). Each SRD is normalized to that animal's mean control SRD to form reflection difference ratios (RDRs).

(2) Quality Control - Hairless guinea pigs must have baseline reflectance readings of less than 13 on the a* chromaticity parameter at each dose site to qualify for treatment. Individuals not meeting this criterion are replaced. SDRs from non-TSP control sites are quality controlled for consistency within separate historical critical limits ($\pm$ 3 standard deviations) previously established by a sample size of no fewer than 24 dose sites. Any animal that does not exhibit control SRDs within the established $\pm$ 3 standard deviation critical limits is eliminated from the database. This procedure attempts to eliminate from the population of hairless guinea pigs those that exhibit unusually low and high degrees of sensitivity to HD vapors at the control site.

There can be no more than two exclusions from each replicate test day due to quality control reasons. Unacceptable replicate test days are repeated.

(3) Data Summary - Univariate statistics on raw SRDs and derived RDRs are calculated and tabulated by treatment. Paired t tests are conducted on pre- and

post-anesthetic reflectance readings, at both unexposed and exposed readings. Multiple comparisons tests are performed to determine ranking of TSPs tested. If a one-way analysis of variance on the data from the dose response study detects a significant dose response, then a probit or other logistic function may be fitted to the data.

(4) Comparison with Other Indices of Irritation - Correlation of RDRs with other indices of irritation is performed to test for their association and to confirm skin reflectance as an acceptable method of estimating irritation.

8. Records to be Maintained:

   A. HD and TSP inventory, specifications, and usage

   B. Dosage preparation and administration

   C. Animal receipt and quarantine records

   D. Animal data from all tests performed

   E. Decontamination results and disposal records

9. Reports:

   A final report is prepared and submitted within 30 days after completion of the task. It includes at least the following:

   A. Signature page for key study individuals and their responsibilities

   B. Experimental design

   C. In vivo test data

   D. Application procedures

   E. Tabulation of response data for each exposure, or for each TSP tested

   F. Statistical methodology used

   G. Discussion

10. Approval Signatures:

Thomas H. Snider, B.S., D.A.B.T.
Study Director

9-3-93
Date

Frances M. Reid, D.V.M., D.A.B.V.T.
Study Advisor

9-3-93
Date

David W. Hobson, Ph.D., D.A.B.T.
Principal Investigator and Manager
Medical Research and Evaluation Facility

9/9/93
Date

Allen G. Manus, D.V.M.
Study Veterinarian

9/16/93
Date

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

30 Sep 93
Date

Efficacy Assessment of Topical Skin Protectants Against
Sulfur Mustard Vapors In Hairless Guinea Pigs

MREF Protocol 99 Amendment No. 1 (deletions are shown as stricken, and additions are shown in bold type)

A. Change: On Page 1, replace Sections 1, 3, and 5 with the following, respectively:

1. Co-Principal Investigator and Manager: **John B. Johnson, D.V.M.,** ~~David W. Hobson, Ph.D., D.A.B.T.~~ , Medical Research and Evaluation Facility (MREF)

3. Study Veterinarian: **Tracy A. Peace** ~~Allen G. Manus~~, D.V.M.

5. Sponsor Monitor: LTC **Richard R. Stotts, D.V.M.** ~~Don W. Korte, Jr., Ph.D.~~, U.S. Army Medical Research Institute of Chemical Defense (USAMRICD

Reason: These substitutions are needed due to personnel changes at the MREF.

Impact: These substitutions will have no impact on the study.

B. Insert: On Page 1, after the last sentence in Section 6, the following:

No portions of this study are conducted under Good Laboratory Practice Regulations.

Reason: Results from this study will not be submitted for review by a government regulatory agency.

Impact: These changes will have no impact on the study.

C. Change: On Page 4, replace Sections 7.A.(7-9) with the following:

(7) Housing - Before being used in experiments, hairless guinea pigs are housed in ~~groups of three in polycarbonate~~ cages equipped with automatic watering systems.

(8) Lighting - Fluorescent lighting, light/dark cycle is 12 hr each per day.

(9) Temperature - Maintained at ***approximately*** *18-26* ***C*** *degrees. At least 90 percent of the total recordings will fall within the specified range.*

Reason: *The hairless guinea pig census may not consistently permit housing in groups of three. The cage racks and cages on hand at the MREF that permit automatic watering* are made of stainless steel. The target temperature range is given on the Celsius scale.

Impact: These changes will have no impact on the study.

D. Change: On Page 5, replace the first sentence of Section 7.A.(15) with the following:

Animal Care During Test - All hairless guinea pigs are held singly in the MREF hood system in polycarbonate cages positioned on pads heated ~~*to a minimum of 41 C*~~ *with* ***warmed*** *circulating water.*

Reason: The circulating bath heater may not be able to attain a water temperature of 41C, however, a holding temperature that maintains the comfort of anesthetized hairless guinea pigs will be standardized after initial studies determine its heating capacity.

Impact: The holding cage floor will be slightly less warm, although still comfortable to the subject.

E. Change: On Page 10, replace Section 7.E.(5) with the following:

Anesthesia - Hairless guinea pigs are given ***approximately*** *6.0 mg/kg (20 mg/mL) xylazine and* ***approximately*** *35.0 mg/kg (100 mg/mL) ketamine* ***(or other, veterinarian-approved anesthetic)*** *by intramuscular injection after baseline reflectance readings are recorded. For TSP efficacy tests, hairless guinea pigs are anesthetized and treated in groups of three, with approximate 30-min intervals between groups. The time of anesthetic administration is recorded. Hairless guinea pigs are then placed in a prone position on holding boards and restrained by taping down each leg with 1/2-inch wide cloth tape. Ketamine anesthetic boosters of the same dose are administered as needed for the entire period of restraint on the boards.* ***The route*** **of these booster administrations may be intramuscular or another, veterinarian-approved route.**

Reason: Anesthetic agents and/or dosages may be altered to reach the desired plane of anesthesia. Previous work has shown that intranasal administration of boosters is effective.

Impact: Handling of the subjects for maintaining anesthesia will be minimized, and technicians may not have to reach over test sites on animals' backs for administering booster injections in the rear legs.

F. Change: On Page 12, replace Section 7.G.(2) with the following:

Applications of HD are made at test-specified times and consist of a constant volume of application. The challenge dose volume may be changed at the direction of the sponsor and/or study director. All safety procedures given in Battelle SOP MREF I-002, "Standard Operating Procedure (SOP) for the Storage, Dilution, and Transfer of GA, GB, GD, GF, TGD, VX, HD, HD/L, and L When CSM Concentration/Quantity is Greater Than Exempt Levels", are observed during handling and dosing of HD. Instructions for applying HD into vapor caps are specified in Battelle SOP MREF II-0093, "Standard Operating Procedure (SOP) for the Percutaneous Application of Either Liquid or Vaporous HD, L, and HL Chemical Surety Materiel to ~~the Dorsum of the Hairless~~ Swine and Guinea Pig ~~to Test Defensive Methods Material~~."

A vapor cap assembly is inverted and placed in a well drilled in an aluminum block. A 10-μL (or other, sponsor-specified) volume of HD is dispensed from a syringe at the center of the paper in the cap. A ~~Hamilton 7001N~~ syringe with a ~~sharp~~blunt-tip, positive displacement needle may be used to provide a point source, air-dropped delivery. A larger syringe may be used in a calibrated micrometer-driven dosing device (MDDD) to administer HD. Alternatively, a calibrated pipettor such as a 100-μL capacity Pipetman may be used with a disposable pipet tip. If a droplet of HD remains on the end of the needle or tip, the needle or tip may be brought down close to the paper surface so as to "wick" off the droplet.

Reason: Changes in the first paragraph were necessitated by title changes resulting from revisions of the standard operating procedures applicable to this study. Changes in the second paragraph are in accord with a general MREF policy of avoiding the use of sharp-tipped needles when administering agent.

Impact: These changes will have no impact on the study except to enhance the safety of personnel.

G. Change: On Page 15, replace Section 7.I.(4) with the following:

Pathology - Histopathological evaluation of exposed skin is performed for the dose response study only. Each animal is anesthetized by halothane ***(or other veterinarian-approved anesthetic)*** *inhalation in a sealed glass container. To prevent recovery, the thoracic cavity is surgically opened on the ventral side. Dermal specimens are collected from the center of each test site. Each specimen is identified by placing it into a labeled jar or cassette. Specimens are identified by task number, charge account number, study director, date and time of tissue harvesting,* ~~*and*~~ *hairless guinea pig number,* ***and test site.*** *A warning label stating that the skin samples were exposed to HD vapors is affixed to the outside of each container and to the outside of the box used for transportation. All samples are retained at the MREF for 24 hr before being* ***opened*** ~~*transported to another facility*~~ *for histologic processing. After fixation, they are processed for routine hematoxylin and eosin staining and histopathologic evaluation by light microscopy. Each specimen is evaluated for microvesication, intracellular edema, pustular epidermatitis, and follicular involvement (necrosis), and receives a score of 1 for the presence of each marker and a 0 for its absence. The histologic slides are sent with the report to the sponsor for confirmation.*

Reasons:

- *Other anesthetics may be more appropriate than halothane.*
- Each sample of dose response study test site skin requires treatment identification that associates it with an exposure period.
- The MREF has acquired the capability to process histologic specimens since the original writing of this protocol. The 24-hr waiting period ensures decontamination of any HD in the tissue sample.

Impact:

- Substituting another inhalation anesthetic for halothane at the time of euthanasia will have no impact on the results of this study.
- Identification of the skin test sites is crucial to correlating effect (histopathologic results) with cause (the test regimen).
- Whether the skin samples are processed at the MREF or another facility will have no impact on this study.

Thomas H. Snider, B.S., D.A.B.T.
Study Director

5-31-95
Date

LTC Richard R. Stotts, COR
USAMRICD

1 JUN 95
Date

Efficacy Assessment of Topical Skin Protectants Against Sulfur Mustard Vapors in Hairless Guinea Pigs

MREF Protocol 99 Amendment No. 2 (deletions are shown as stricken, and additions are shown in bold type)

A. Change: On Page 3, replace Sections 7.A.(2) and 7.A.(5) with the following, respectively:

(2) Weight Range - **preferably** 300 to 400 grams. **Hairless guinea pigs from 275 to 425 g may be used at the discretion of the Study Director, if hairless guinea pigs in the preferred range are not available.**

Reason: Research animals in new environments do not always gain weight at the expected rate. Allowing for a slightly wider weight range of acceptance may preclude the rejection of such animals from placement on study. This may save time and support resources that otherwise would be spent by having to wait on smaller animals to gain weight, or waiting on replacements for individuals that have grown beyond the acceptable range.

Impact: The susceptibility of hairless guinea pigs to the dermatotoxic effects of HD may be linked to body weight, owing to the degree of epidermal cornification and the barrier it provides. However, a 25-g difference in the weight of a small number of individuals is not expected to significantly impact the results of this study.

Thomas H. Snider, B.S., D.A.B.T.
Study Director

8/21/95
Date

LTC Richard R. Stotts, COR

21 Aug 95
Date

APPENDIX B

Figures

Figure 1. Control Chart of HD Doses Applied onto HGP Dose Sites, with 95 Percent Confidence Limits

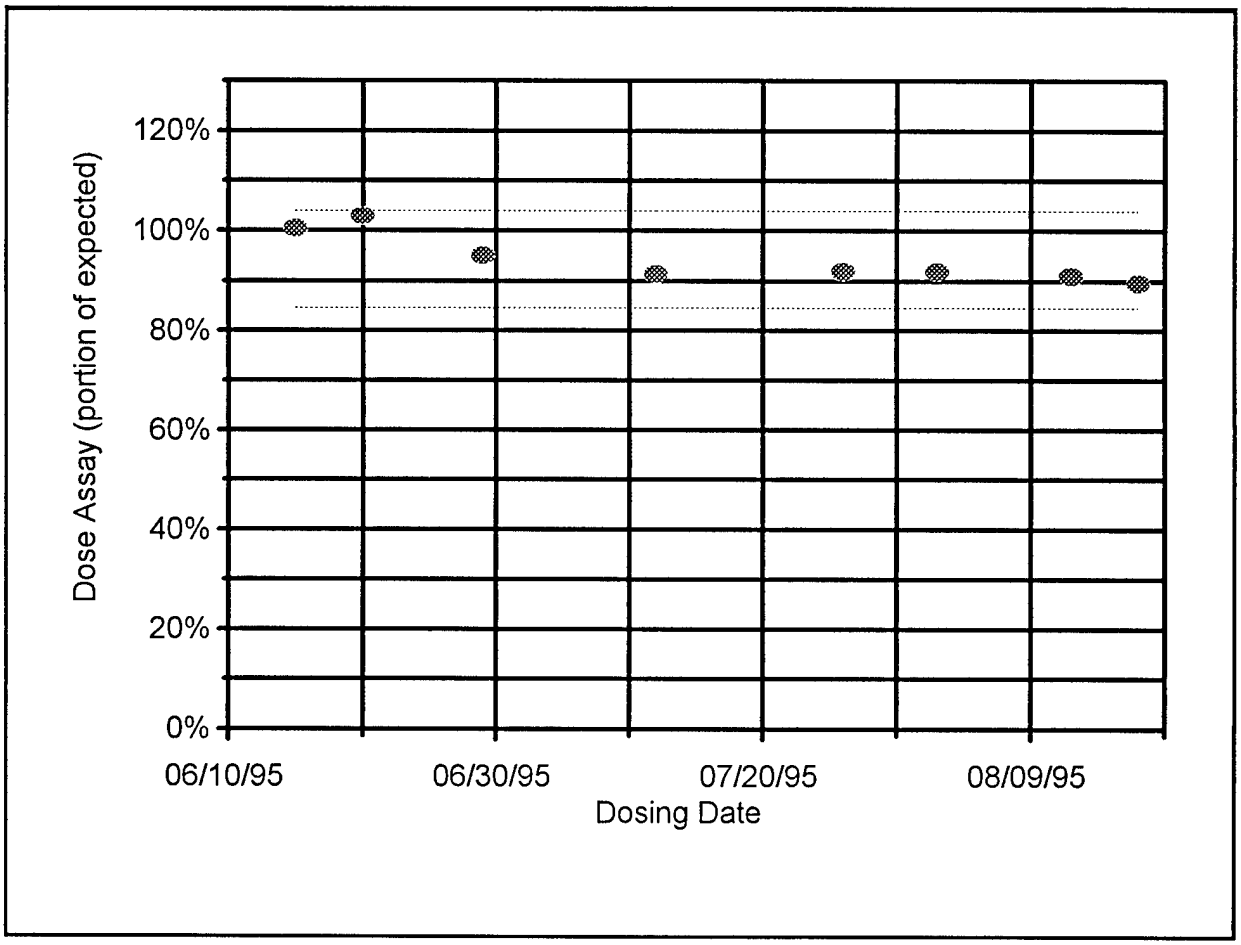


Figure 2. Percent Incidence of Four Histopathological Endpoints: Observed and Smoothed Percentages Versus HD Vapor Exposure Time

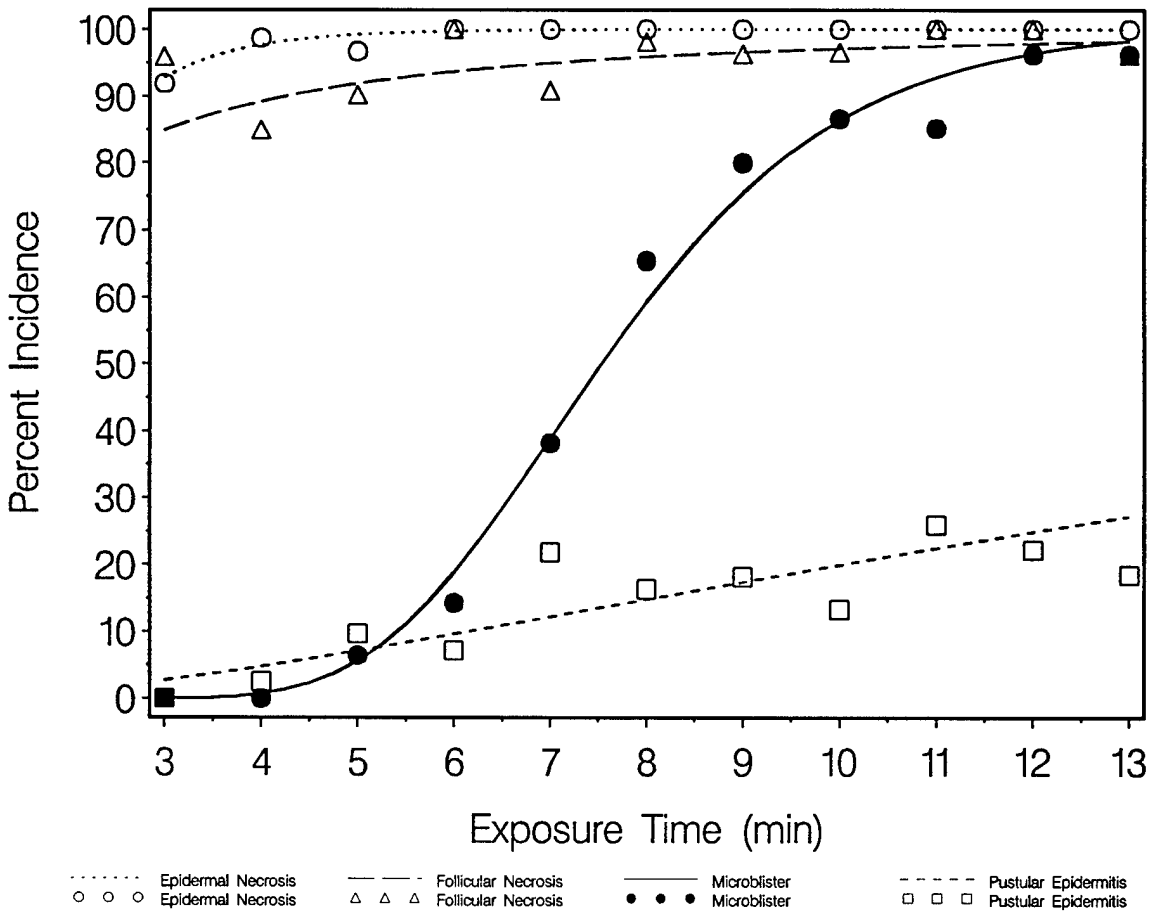


Figure 3. Average Observed Severity Scores of Four Histopathological Endpoints Versus HD Vapor Exposure Times

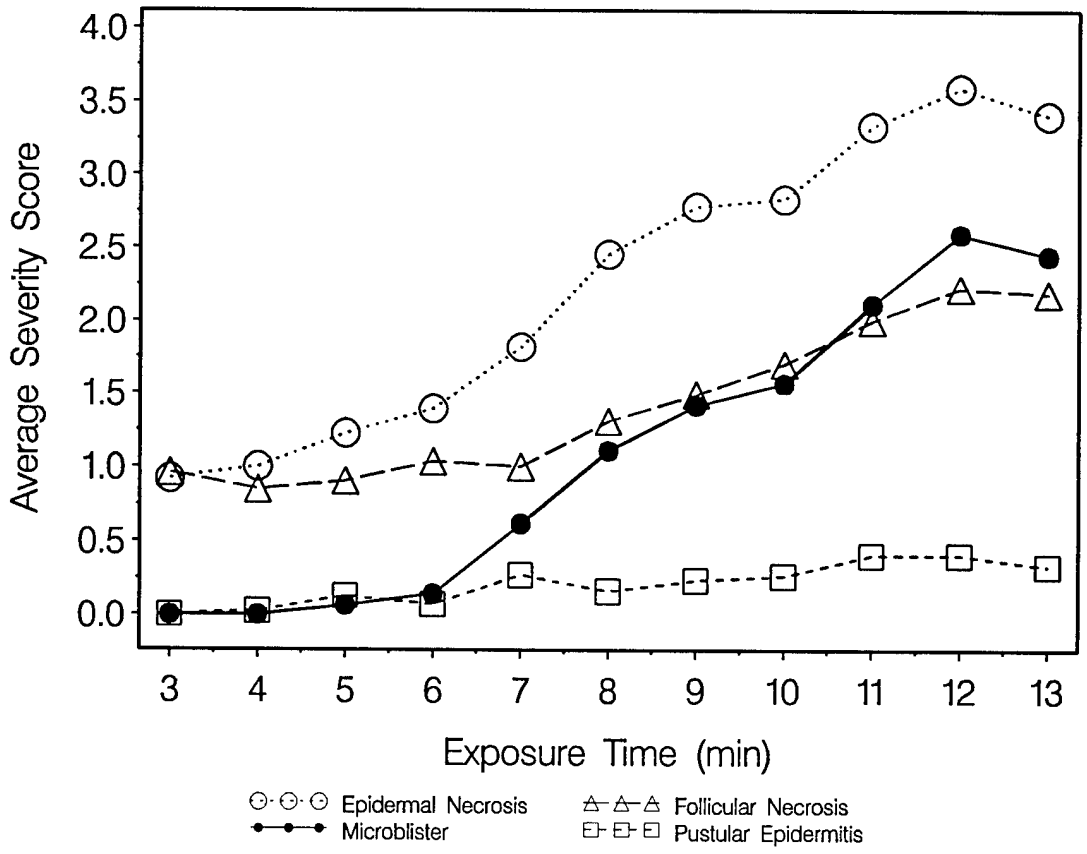


Figure 4. Observed and Modeled Percent Incidence of Microblisters for MREF Task 92-29 Data

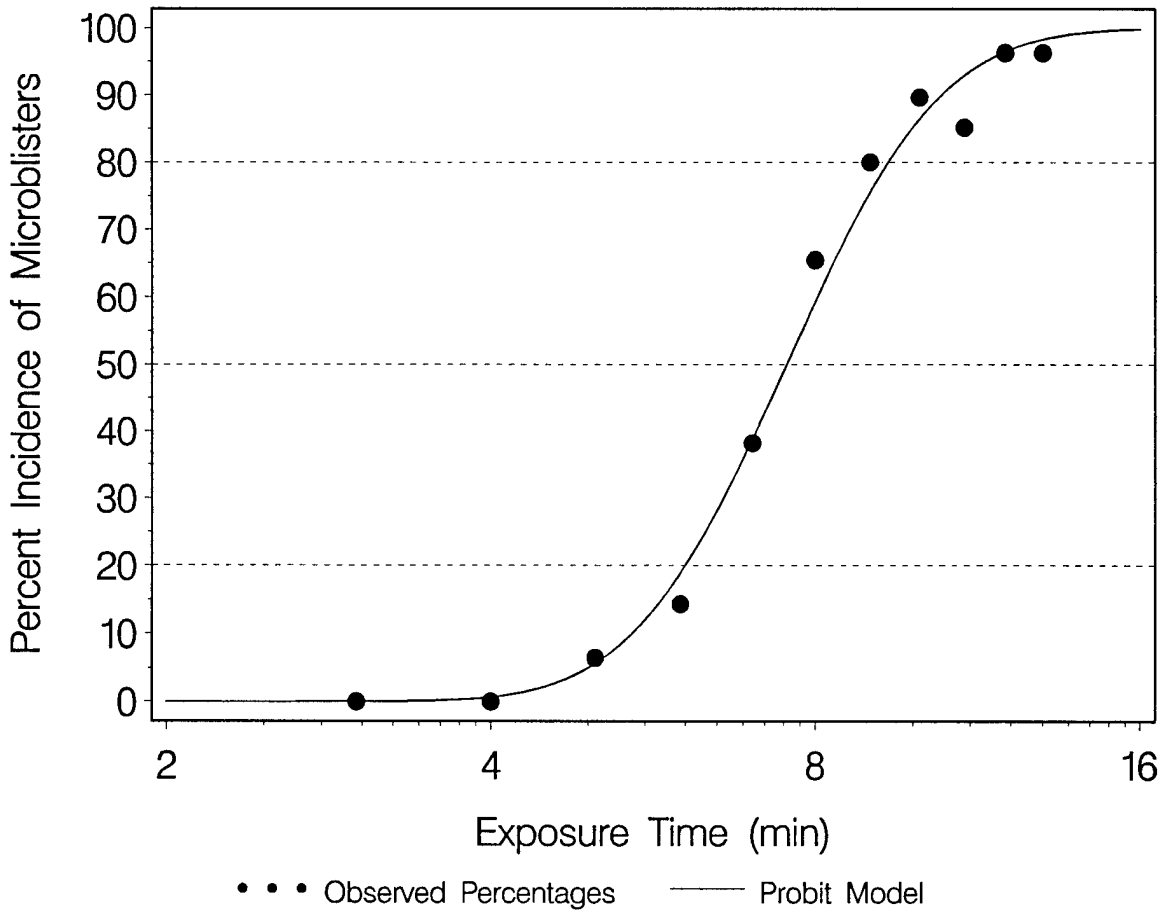


Figure 5. Observed and Modeled Percent Incidence of Microblisters for MRICD Study Data.

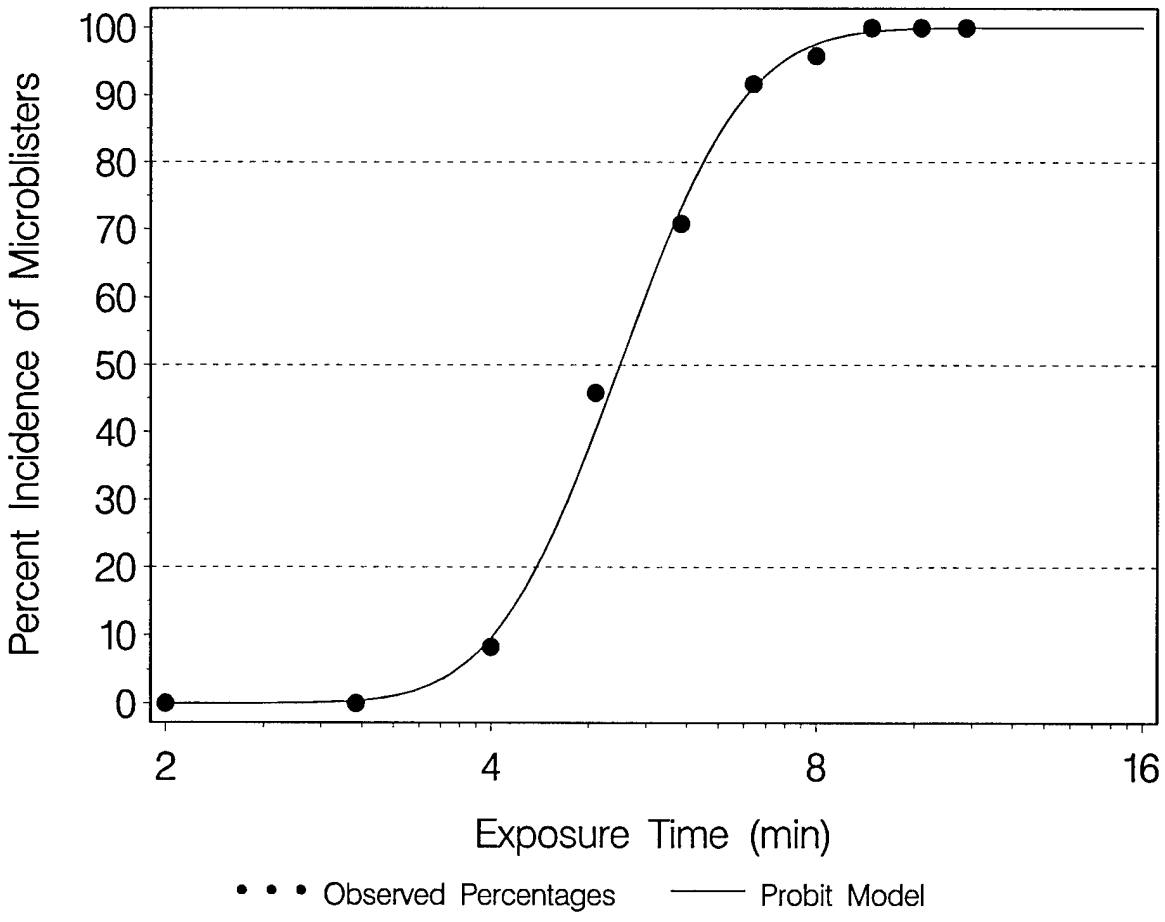
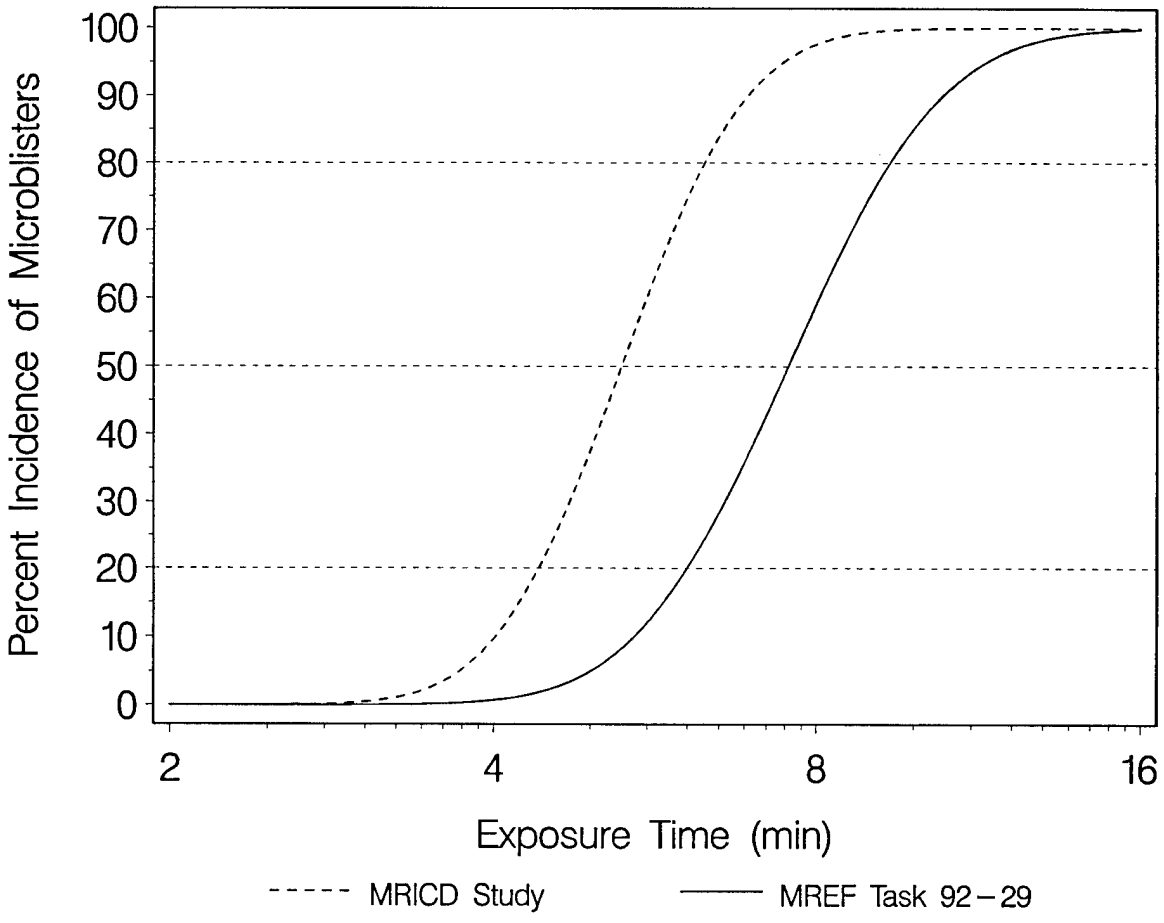


Figure 6. Modeled Percent Incidence of Microblisters for USAMRICD Study Data and MREF Task 92-29 Data



APPENDIX C

Tables

Table 1. Raw Data for Skin Reflectance Readings on Test Day 1, 6/14/95

HGP Reflectance, Red (a*) Scale				
Before Anesthesia				
Animal	Site			Site
3106	B	10.27	10.03	A
	D			C
	F	7.53	8.94	E
	H			G
3104	B	11.30	13.62	A
	D			C
	F	8.75	10.66	E
	H			G
3111	B	11.80	8.90	A
	D			C
	F	8.20	10.32	E
	H			G
3102	B	9.80	9.62	A
	D			C
	F	8.46	7.81	E
	H			G
3103	B	8.41	10.25	A
	D			C
	F	7.31	8.39	E
	H	7.85	7.55	G
3108	B	10.85	11.73	A
	D			C
	F	9.59	10.70	E
	H	8.51	8.78	G
3107	B	13.13	13.16	A
	D			C
	F	10.50	10.39	E
	H	9.74	10.92	G

Table 2. Raw Data for Skin Reflectance Readings on Test Day 2, 6/20/95

HGP Reflectance, Red (a*) Scale												
	Before Anesthesia				After Ketamine Anesthesia				Skin Reflectance Relative Change			
Animal	Site			Site	Site			Site	Site			Site
3120	B	8.50	11.71	A	B	4.96	4.85	A	B	0.35	0.68	A
	D			C	D			C	D			C
	F	9.44	11.98	E	F	5.39	5.14	E	F	0.38	0.64	E
	H	12.59	13.09	G	H	4.89	6.29	G	H	0.60	0.53	G
3115	B	14.11	14.47	A	B	6.05	6.62	A	B	0.56	0.55	A
	D			C	D			C	D			C
	F	8.53	12.37	E	F	6.91	7.48	E	F	0.16	0.47	E
	H	10.24	9.68	G	H	4.71	5.82	G	H	0.56	0.39	G
3118	B	12.23	10.82	A	B	5.90	6.24	A	B	0.55	0.40	A
	D			C	D			C	D			C
	F	12.10	10.94	E	F	6.16	5.55	E	F	0.52	0.47	E
	H	9.67	8.91	G	H	5.39	4.93	G	H	0.46	0.43	G
3121	B	13.80	11.73	A	B	6.79	8.04	A	B	0.55	0.29	A
	D			C	D			C	D			C
	F	10.64	9.68	E	F	5.02	5.12	E	F	0.55	0.45	E
	H			G	H	5.10	4.91	G	H			G
3122	B	11.41	11.22	A	B	6.37	6.10	A	B	0.45	0.45	A
	D			C	D			C	D			C
	F	9.92	9.53	E	F	6.32	5.58	E	F	0.37	0.41	E
	H	11.63	11.62	G	H	6.04	5.71	G	H	0.48	0.51	G
3116	B	12.34	13.17	A	B	7.77	5.77	A	B	0.36	0.58	A
	D			C	D			C	D			C
	F	10.83	11.70	E	F	3.93	5.06	E	F	0.61	0.59	E
	H	11.92	9.97	G	H		5.30	G	H		0.43	G
3117	B	10.23	12.62	A	B	7.44	7.54	A	B	0.24	0.44	A
	D			C	D			C	D			C
	F	7.16	8.11	E	F	5.02	4.92	E	F	0.28	0.42	E
	H	10.51	8.86	G	H	4.87	5.43	G	H	0.58	0.35	G

Table 3. Raw Data for Skin Reflectance Readings on Test Day 3, 6/29/95

HGP Reflectance, Red (a*) Scale

	Before Anesthesia				After Ketamine Anesthesia				Skin Reflectance Relative Change			
Animal	Site			Site	Site			Site	Site			Site
3126	B	10.41	10.10	A	B	8.38	5.88	A	B	0.20	0.41	A
	D			C	D			C	D			C
	F	8.52	9.28	E	F	5.18	7.22	E	F	0.38	0.23	E
	H	9.23	8.68	G	H	7.03	6.34	G	H	0.25	0.26	G
3134	B	10.81	8.72	A	B	6.43	6.42	A	B	0.45	0.24	A
	D			C	D			C	D			C
	F	9.39	9.22	E	F	5.97	6.08	E	F	0.37	0.34	E
	H	9.15	10.35	G	H	5.66	5.18	G	H	0.36	0.53	G
3130	B	11.52	12.26	A	B	5.78	6.39	A	B	0.48	0.49	A
	D			C	D			C	D			C
	F	9.91	9.44	E	F	6.52	6.97	E	F	0.35	0.26	E
	H	11.30	10.75	G	H	5.29	4.22	G	H	0.55	0.59	G
3131	B	10.51	8.84	A	B	6.00	6.05	A	B	0.47	0.29	A
	D			C	D			C	D			C
	F	10.10	7.20	E	F	5.26	5.89	E	F	0.56	0.15	E
	H	9.43	9.87	G	H	4.41	4.12	G	H	0.52	0.60	G
3129	B	8.93	8.97	A	B	6.78	5.94	A	B	0.24	0.34	A
	D			C	D			C	D			C
	F	6.77	7.82	E	F	4.35	4.58	E	F	0.33	0.44	E
	H	6.37	5.84	G	H	3.90	3.78	G	H	0.40	0.34	G
3135	B	8.11	10.08	A	B	4.56	6.57	A	B	0.39	0.39	A
	D			C	D			C	D			C
	F	8.00	8.34	E	F	4.78	4.17	E	F	0.39	0.51	E
	H	7.51	7.65	G	H	5.04	4.24	G	H	0.33	0.45	G
3132	B	10.47	10.98	A	B	6.27	5.95	A	B	0.39	0.47	A
	D			C	D			C	D			C
	F	9.68	8.38	E	F	5.90	5.53	E	F	0.42	0.32	E
	H	6.13	8.22	G	H	4.35	4.17	G	H	0.25	0.56	G

Table 4. Raw Data for Skin Reflectance Readings on Test Day 4, 7/12/95

HGP Reflectance, Red (a*) Scale												
	Before Anesthesia				After Ketamine Anesthesia				Skin Reflectance Relative Change			
Animal	Site			Site	Site			Site	Site			Site
3142	B	13.81	14.12	A	B	8.01	7.35	A	B	0.42	0.48	A
	D			C	D			C	D			C
	F	10.28	10.41	E	F	5.25	4.86	E	F	0.49	0.54	E
	H	10.58	11.12	G	H	5.28	5.16	G	H	0.49	0.55	G
3139	B	14.19	11.89	A	B	8.63	7.13	A	B	0.43	0.37	A
	D			C	D			C	D			C
	F	11.54	10.19	E	F	5.77	5.30	E	F	0.53	0.45	E
	H	11.15	10.90	G	H	5.41	5.87	G	H	0.52	0.46	G
3145	B	13.49	10.76	A	B	6.89	6.96	A	B	0.54	0.31	A
	D			C	D			C	D			C
	F	10.45	10.33	E	F	4.84	5.17	E	F	0.54	0.50	E
	H	11.30	10.21	G	H	5.75	4.88	G	H	0.52	0.50	G
3144	B	13.04	12.66	A	B	8.06	7.81	A	B	0.39	0.38	A
	D			C	D			C	D			C
	F	11.09	10.75	E	F	5.35	6.81	E	F	0.53	0.36	E
	H	12.05	11.10	G	H	6.51	6.08	G	H	0.48	0.43	G
3140	B	12.31	13.15	A	B	8.73	6.45	A	B	0.28	0.53	A
	D			C	D			C	D			C
	F	9.84	9.56	E	F	5.31	5.34	E	F	0.47	0.44	E
	H	11.12	10.33	G	H	4.61	4.93	G	H	0.61	0.50	G
3138	B	10.97	13.61	A	B	8.50	7.80	A	B	0.20	0.47	A
	D			C	D			C	D			C
	F	9.55	10.11	E	F	4.50	5.27	E	F	0.51	0.49	E
	H	10.49	9.44	G	H	5.06	5.50	G	H	0.54	0.40	G
3141	B	13.76	11.96	A	B	7.89	8.12	A	B	0.46	0.30	A
	D			C	D			C	D			C
	F	9.46	8.99	E	F	4.34	4.91	E	F	0.56	0.44	E
	H	11.76	9.84	G	H	5.85	5.44	G	H	0.55	0.41	G

Table 5. Raw Data for Skin Reflectance Readings on Test Day 5, 7/26/95

HGP Reflectance, Red (a*) Scale

	Before Anesthesia				After Ketamine Anesthesia				Skin Reflectance Relative Change			
Animal	Site			Site	Site			Site	Site			Site
3146	B	10.35	11.61	A	B	6.52	7.34	A	B	0.35	0.39	A
	D			C	D			C	D			C
	F	10.78	9.53	E	F	5.64	5.54	E	F	0.51	0.39	E
	H	9.40	10.05	G	H	4.98	5.32	G	H	0.45	0.49	G
3148	B	12.12	12.91	A	B	6.38	6.91	A	B	0.46	0.48	A
	D			C	D			C	D			C
	F	10.85	12.18	E	F	5.71	6.23	E	F	0.45	0.52	E
	H	8.93	8.49	G	H	4.70	4.72	G	H	0.49	0.43	G
3150	B	10.89	10.58	A	B	6.26	6.49	A	B	0.43	0.38	A
	D			C	D			C	D			C
	F	9.87	8.83	E	F	5.93	5.32	E	F	0.42	0.38	E
	H	8.89	9.17	G	H	4.71	4.63	G	H	0.46	0.50	G
3153	B	10.73	12.27	A	B	6.86	8.70	A	B	0.34	0.31	A
	D			C	D			C	D			C
	F	10.92	12.37	E	F	6.67	6.43	E	F	0.36	0.51	E
	H	10.70	8.10	G	H	6.10	4.16	G	H	0.49	0.42	G
3152	B	9.41	10.12	A	B	6.17	7.03	A	B	0.33	0.32	A
	D			C	D			C	D			C
	F	7.93	6.78	E	F	5.05	5.56	E	F	0.39	0.17	E
	H	8.37	9.38	G	H	6.90	6.99	G	H	0.17	0.27	G
3151	B	10.92	8.84	A	B	5.67	5.72	A	B	0.53	0.32	A
	D			C	D			C	D			C
	F	6.75	7.31	E	F	5.55	5.36	E	F	0.17	0.28	E
	H	7.67	8.02	G	H	5.07	5.20	G	H	0.33	0.36	G
3149	B	11.88	10.57	A	B	6.91	5.82	A	B	0.44	0.42	A
	D			C	D			C	D			C
	F	10.43	9.62	E	F	4.94	4.82	E	F	0.55	0.48	E
	H	10.57	10.62	G	H	5.54	4.52	G	H	0.47	0.58	G

Table 6. Raw Data for Skin Reflectance Readings on Test Day 6, 8/02/95

HGP Reflectance, Red (a*) Scale												
	Before Anesthesia				After Ketamine Anesthesia				Skin Reflectance Relative Change			
Animal	Site			Site	Site			Site	Site			Site
3156	B	12.61	12.55	A	B	9.35	9.54	A	B	0.26	0.24	A
	D			C	D			C	D			C
	F	11.86	10.09	E	F	6.25	6.30	E	F	0.51	0.35	E
	H	11.09	10.56	G	H	6.86	6.82	G	H	0.39	0.35	G
3154	B	12.87	12.82	A	B			A	B			A
	D			C	D			C	D			C
	F	10.66	10.76	E	F			E	F			E
	H	10.54	10.01	G	H			G	H			G
3147	B	11.37	12.95	A	B	6.75	6.86	A	B	0.38	0.50	A
	D			C	D			C	D			C
	F	9.95	9.65	E	F	5.26	5.79	E	F	0.48	0.39	E
	H	11.24	10.11	G	H	6.42	6.36	G	H	0.45	0.35	G
3155	B	12.83	12.03	A	B	6.85	6.94	A	B	0.48	0.41	A
	D			C	D			C	D			C
	F	12.48	11.53	E	F	5.87	6.41	E	F	0.55	0.43	E
	H	11.16	12.73	G	H	7.18	6.15	G	H	0.33	0.55	G
3157	B	11.78	11.58	A	B	5.99	6.63	A	B	0.50	0.42	A
	D			C	D			C	D			C
	F	10.40	10.68	E	F	6.38	6.29	E	F	0.38	0.42	E
	H	9.03	9.57	G	H	5.66	5.78	G	H	0.36	0.41	G
3160	B	12.18	11.33	A	B	6.56	6.32	A	B	0.48	0.43	A
	D			C	D			C	D			C
	F	11.27	12.44	E	F	4.75	6.34	E	F	0.55	0.51	E
	H	11.38	11.37	G	H	5.06	5.81	G	H	0.56	0.49	G
3161	B	11.25	12.08	A	B	6.81	7.94	A	B	0.38	0.35	A
	D			C	D			C	D			C
	F	12.95	12.22	E	F	6.54	7.27	E	F	0.51	0.39	E
	H	7.85	8.65	G	H	5.64	5.37	G	H	0.27	0.40	G

Table 7. Raw Data for Skin Reflectance Readings on Test Day 7, 8/14/95

HGP Reflectance, Red (a*) Scale												
	Before Anesthesia				After Ketamine Anesthesia				Skin Reflectance Relative Change			
Animal	Site			Site	Site			Site	Site			Site
3158	B	10.84	9.11	A	B	7.86	7.96	A	B	0.30	0.12	A
	D			C	D			C	D			C
	F	7.81	7.45	E	F	5.53	5.92	E	F	0.30	0.20	E
	H	6.78	11.51	G	H	5.70	6.40	G	H	0.12	0.56	G
3169	B	10.05	11.16	A	B	8.10	7.49	A	B	0.18	0.35	A
	D			C	D			C	D			C
	F	11.37	12.38	E	F	5.95	6.36	E	F	0.46	0.51	E
	H	11.56	10.82	G	H	5.37	7.14	G	H	0.55	0.33	G
3162	B	12.45	12.99	A	B	7.96	7.31	A	B	0.35	0.45	A
	D			C	D			C	D			C
	F	12.24	12.38	E	F	7.14	7.92	E	F	0.41	0.36	E
	H	12.13	12.61	G	H	6.78	6.32	G	H	0.43	0.51	G
3159	B	11.35	12.05	A	B	6.22	7.69	A	B	0.44	0.37	A
	D			C	D			C	D			C
	F	11.32	9.25	E	F	6.69	6.34	E	F	0.45	0.28	E
	H	11.46	10.34	G	H	6.75	5.31	G	H	0.43	0.46	G
3163	B	12.75	12.51	A	B	7.30	6.10	A	B	0.43	0.51	A
	D			C	D			C	D			C
	F	11.10	10.85	E	F	5.21	5.63	E	F	0.54	0.48	E
	H	11.60	11.56	G	H	5.52	5.96	G	H	0.53	0.48	G
3167	B	10.01	7.94	A	B	5.00	4.24	A	B	0.56	0.41	A
	D			C	D			C	D			C
	F	8.72	8.10	E	F	3.66	4.17	E	F	0.60	0.47	E
	H	7.13	7.98	G	H	3.85	2.96	G	H	0.43	0.66	G
3165	B	11.64	10.79	A	B	7.08	8.00	A	B	0.41	0.25	A
	D			C	D			C	D			C
	F	11.82	11.23	E	F	6.43	7.06	E	F	0.47	0.36	E
	H	10.09	10.56	G	H	9.77	6.49	G	H	0.03	0.39	G

Table 8. Raw Data for Skin Reflectance Readings on Test Day 8,8/17/95

HGP Reflectance, Red (a*) Scale												
	Before Anesthesia				After Ketamine Anesthesia				Skin Reflectance Relative Change			
Animal	Site			Site	Site			Site	Site			Site
3179	B	10.87	12.40	A	B	6.76	6.04	A	B	0.35	0.55	A
	D			C	D			C	D			C
	F	11.56	11.56	E	F	5.46	5.72	E	F	0.53	0.51	E
	H	8.81	10.09	G	H	4.11	4.66	G	H	0.50	0.57	G
3178	B	12.35	11.68	A	B	5.83	5.32	A	B	0.54	0.53	A
	D			C	D			C	D			C
	F	9.23	9.20	E	F	4.80	4.95	E	F	0.48	0.46	E
	H	10.69	10.08	G	H	6.01	5.08	G	H	0.45	0.48	G
3180	B	11.24	10.20	A	B	5.70	5.50	A	B	0.52	0.44	A
	D			C	D			C	D			C
	F	11.12	10.41	E	F	5.30	4.95	E	F	0.54	0.51	E
	H	7.47	7.78	G	H	4.67	3.84	G	H	0.37	0.52	G
3176	B	12.10	12.69	A	B	7.13	6.71	A	B	0.40	0.48	A
	D			C	D			C	D			C
	F	9.14	9.22	E	F	5.20	4.53	E	F	0.43	0.51	E
	H	9.50	9.80	G	H	4.66	4.64	G	H	0.50	0.53	G
3173	B	12.42	11.45	A	B	7.09	5.69	A	B	0.45	0.48	A
	D			C	D			C	D			C
	F	10.41	8.84	E	F	5.93	5.47	E	F	0.47	0.35	E
	H	8.54	7.90	G	H	4.99	4.51	G	H	0.43	0.41	G
3168	B	10.88	9.30	A	B	6.13	5.57	A	B	0.47	0.37	A
	D			C	D			C	D			C
	F	8.65	7.81	E	F	3.76	5.28	E	F	0.59	0.31	E
	H	8.70	7.87	G	H	5.18	4.84	G	H	0.42	0.37	G
3171	B	12.75	12.70	A	B	7.65	7.31	A	B	0.40	0.42	A
	D			C	D			C	D			C
	F	12.63	11.56	E	F	6.52	6.08	E	F	0.51	0.45	E
	H	12.82	11.40	G	H	6.39	8.83	G	H	0.53	0.21	G

Table 9. Incidence of Test Sites That Were Scored Positive for Each Histopathologic Endpoint

Exposure Period (min)	Number of Sites	Histopathologic Endpoint						
		Microblistering	Epidermal necrosis	Follicular Necrosis	Pustular Epidermitis	Dermal Necrosis	Hemorrhage	Vascular Necrosis
		Incidence (Percent)						
0	55	0	7	2	0	0	0	0
3	25	0	92	96	0	0	0	0
4	80	0	99	85	3	0	0	0
5	31	6	97	90	10	0	0	0
6	28	14	100	100	7	0	0	0
7	55	38	100	91	22	0	2	0
8	55	65	100	98	16	7	4	0
9	55	80	100	96	18	4	9	0
10	30	87	100	97	13	3	13	0
11	27	85	100	100	26	11	11	0
12	27	96	100	100	22	11	33	0
13	27	96	100	96	19	15	15	0

Table 10. Average Severity Scores for Histopathologic Endpoints

Exposure Period (min)	Number of Sites	Histopathologic Endpoint						
		Microblistering	Epidermal necrosis	Follicular Necrosis	Pustular Epidermitis	Dermal Necrosis	Hemorrhage	Vascular Necrosis
		Endpoint Severity (0 to 4)						
0	55	0.0	0.1	0.0	0.0	0.0	0.0	0.0
3	25	0.0	0.9	1.0	0.0	0.0	0.0	0.0
4	80	0.0	1.0	0.9	0.0	0.0	0.0	0.0
5	31	0.1	1.2	0.9	0.1	0.0	0.0	0.0
6	28	0.1	1.4	1.0	0.1	0.0	0.0	0.0
7	55	0.6	1.8	1.0	0.3	0.0	0.0	0.0
8	55	1.1	2.5	1.3	0.2	0.1	0.0	0.0
9	55	1.4	2.8	1.5	0.2	0.0	0.1	0.0
10	30	1.6	2.8	1.7	0.3	0.1	0.2	0.0
11	27	2.1	3.3	2.0	0.4	0.2	0.1	0.0
12	27	2.6	3.6	2.2	0.4	0.1	0.3	0.0
13	27	2.4	3.4	2.2	0.3	0.2	0.1	0.0

Table 11. Summary of Results of Probit Dose-Response Model on Percent Incidence of Microblisters from Two Studies

Parameter	Estimate	Lower 95% Confidence Limit	Upper 95% Confidence Limit
Task 92-29 Studies			
Slope[a]	8.94	7.41	10.50
ED_{20}	6.06	5.66	6.40
ED_{50}	7.53	7.21	7.85
ED_{80}	9.35	8.92	9.91
Studies Performed at USAMRICD			
Slope[a]	10.90	8.93	12.90
ED_{20}	4.41	4.12	4.65
ED_{50}	5.27	5.03	5.49
ED_{80}	6.29	6.02	6.63

[a] The model was fitted to a logarithm base 10 of HD vapor exposure periods.